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Dendritic spine density in the default mode network: a postmortem morphometric pilot study of schizophrenia

Master's thesis in science, Neuroscience

Oslo, May 2016

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Acknowledgements

This thesis is part of a two-year program of Master of Science in Neuroscience, performed in collaboration with the Norwegian University of Science and Technology and the University of Oslo.

I will thank Oslo University Hospital and Ole A. Andreassen for giving me the opportunity to work on this project. I would also like to direct a special gratitude to Kjell Martin Moksnes for his helpful work. I will also thank the people who have been vital in the creation of this thesis.

First of all, I will thank Svend Davanger, my principal supervisor, for his insightful comments during the process of writing this thesis.

Laura Marian Valencia Pesqueira, fellow master student and Golgi staining expert; and Marianne Vaadal, lab engineer: thank you for patiently helping me in the lab.

Torstein Landaas Skjervold, “late night labing”-partner, thank you for your cheerful spirits in the office.

Finally, I want to thank my dear twin sister, Charlotte Kjøge Wilhelmsen, for proofreading my thesis and for all her support throughout the creation of this thesis.

Oslo, May 2016
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Abstract

Neuroimaging studies have found decreased activity and functional connectivity within and between brain regions in subjects with schizophrenia. These findings correlate with a decline in dendritic spine density in the corresponding areas, especially in prefrontal regions. Schizophrenia is therefore generally associated with a decrease in functional and structural connectivity. However, in regions associated with the default mode network (DMN) subjects with schizophrenia consistently demonstrate abnormally increased activity and internal functional connectivity, disagreeing with the notion that schizophrenia is a disconnection syndrome. The structural basis for this hyperactivity and hyperconnectivity remains uncertain. The objective of the present study was to investigate structural connectivity at the synaptic level in the main regions of the DMN. Dendritic spine density on Golgi-impregnated layer III pyramidal neurons in the functional hubs of the DMN, the medial prefrontal cortex (MPFC) and the posterior cingulate cortex (PCC), from medication-naïve schizophrenic subjects was examined. The results of the study indicated regional variations in spine density within the DMN, with the MPFC showing significantly higher spine density compared to the PCC. As opposed to other studies on healthy subjects, the results of the study indicated that dendritic spine density was abnormally increased in the DMN in schizophrenia. The findings of the present study suggest that the increased activity and functional connectivity of the DMN is coupled with increased structural connectivity.

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List of abbreviations

APA	American psychiatric association
BA	Brodmann area
BOLD	Blood-oxygen-level dependent
CT	Computed tomography
DLPFC	Dorsolateral prefrontal cortex
DMN	Default mode network
DSM-V	Diagnostic and statistical manual of mental disorders, fifth edition
DTI	Diffusion tensor imaging
fMRI	Functional magnetic resonance imaging
ICD	International classification of diseases
MPFC	Medial prefrontal cortex
PCC	Posterior cingulate cortex
PFC	Prefrontal cortex
PMI	Post mortem interval
PSD	Post synaptic density
rCBF	Regional cerebral blood flow
TPN	Task positive network

Introduction

Schizophrenia is a devastating and complex mental illness with a prevalence approaching 1% internationally (Janoutova et al., 2016). According to the Diagnostic and statistical manual of mental disorders, fifth edition (DSM-V), schizophrenia involves a range of behavioral, cognitive and emotional dysfunctions with no single symptom being pathognomonic for the disease (American psychiatric association (APA), 2013). Due to the variation in symptoms observed across cases, schizophrenia is regarded as a heterogeneous syndrome and a spectrum disorder. The symptoms of schizophrenia are commonly grouped into three classes: positive, negative and cognitive. Positive symptoms include phenomena that healthy people usually do not experience and may manifest as hallucinations, delusions, disorganized speech and behavior. These symptoms are commonly considered as the first-rank symptoms. Negative symptoms are often described as the absence of normal behavior and include blunted emotional responses, social isolation and lack of motivation. Finally, cognitive symptoms encompass attentional deficits, poor executive functioning and disruptions to working memory (APA, 2013).

The notion that schizophrenia is a brain disorder dates back to the earliest conceptualizations of the disease. Bleuler, who coined the term schizophrenia ("splitting of the brain") in 1911, emphasized fragmentation of different brain faculties as the cardinal pathology and believed that the origin of these abnormalities could lie in the architecture of the brain (Moskowitz and Heim, 2001). During the last century, advances in technology and the advent of neuroimaging techniques such as computed tomography (CT) and functional magnetic resonance imaging (fMRI) have further furnished the idea of schizophrenia as a disease of the brain. Nevertheless, the exact neurobiological mechanisms remain obscure. The broad range of clinical symptoms in schizophrenia implicates that multiple brain regions might be involved. One of the regions that have been accepted as being central to the pathophysiology of schizophrenia is the prefrontal cortex (PFC) (Goldman-Rakic and Selemon, 1997). Several of the cognitive abilities that are compromised in schizophrenia depend on the integrity of the frontal lobes. In line with the aforementioned view that schizophrenia reflects malfunction in distributed neural circuits, the PFC has widespread reciprocal connections throughout the brain (Yeterian et al., 2012). A major finding to emerge from functional imaging studies of subjects with schizophrenia is that of hypofrontality (Liddle et al., 1992, Andreasen et al., 1992, Andreasen et al., 1997).

Hypofrontality may be defined as a state of reduced cerebral blood flow in the PFC reflecting decreased activation of this region during performance of a broad range of tasks (Ingvar and Frantzen, 1974). Furthermore, patients with schizophrenia also demonstrate reduced functional connectivity within and between different brain regions (Meyer-Lindenberg et al., 2001, Kim et al., 2003, Frith et al., 2005). Functional connectivity refers to the temporal correlation between two neurophysiological events (Friston, 1993).

In addition to the functional abnormalities presented by neuroimaging data, a number of neuropathological features have been identified in schizophrenia, ranging from macroscopic brain alterations (Johnstone et al., 1979) to abnormal neuronal morphology (Garey et al., 1998, Glantz and Lewis, 2000). Most notably, a significant reduction in neuropil in prefrontal regions has been observed in post mortem studies (Selemon et al., 1995, Selemon et al., 1998). These morphometric alterations might reflect synaptic reorganization and are suggestive of aberrant neural connectivity. The relationship between structural features and functional abnormalities is still unclear, however it is hypothesized that the neuropathological findings represent the anatomical substrate of the functional disruptions in brain activity and connectivity present in subjects with schizophrenia. Moreover, these observations implicate that *reduced* functional and anatomical connectivity within and between brain regions are characteristic of the pathology of schizophrenia.

The vast majority of fMRI studies on brain connectivity in schizophrenia point towards both hypoactivity and hypoconnectivity; however, there are some incongruent findings. A relatively new concept emerging from resting state fMRI is that of the default mode network (DMN) (Raichle et al., 2001). The DMN is responsible for spontaneous, self-referential cognition, and has been shown to be dysfunctional in a number of neuropsychiatric diseases ranging from depression and anxiety, to autism spectrum disorder (ASD) and schizophrenia. In schizophrenia, the DMN displays higher activity and functional connectivity compared to healthy control subjects (Whitfield-Gabrieli et al., 2009, Mannell et al., 2010, Guo et al., 2015). DMN has been shown to interact with the networks underlying working memory (Fox et al., 2005) and may thus partly account for the working memory disruptions commonly observed in schizophrenia. Studies on healthy subjects have demonstrated a strong function-structure relationship in connectivity in the DMN (Greicius et al., 2009, Horn et al., 2014). Nevertheless, the structural correlate for the abnormal pattern of activity and functional connectivity of the DMN in schizophrenia remains uncertain.

Background

Information processing in the cerebral cortex

In order to understand how compromised brain connectivity might underlie the clinical symptoms of schizophrenia, a general account of how information processing emerges from interactive neural networks in the brain is necessary. The following sections will review how connectivity in the healthy brain transpires, and how cytoarchitectural features provide an anatomical framework in which communication between neuronal populations may occur. Furthermore, brain connectivity will be discussed in light of the structural and functional abnormalities associated with schizophrenia.

Brain connectivity has several modes and may refer to either anatomical links, or statistical dependencies between neurons and neuronal populations, or between distinct brain regions. The former relates to structure and is subserved by synapses and axonal fiber tracts, while the latter may refer to the temporal correlation of two spatially remote neurophysiological events; i.e., functional connectivity (Friston, 1993). Structural connectivity may be probed at micro scale by assessing synaptic connections that link individual neurons, or at a macro scale. Diffusion tensor imaging (DTI) is an application of MRI enabling fiber tracking, a noninvasive method for assessing whole-brain anatomical connectivity *in vivo*. The main focus in the present thesis will be on structural connectivity at the micro scale, i.e. synaptic connectivity.

Cognitive functions such as language, perception and memory were once hypothesized to arise from local domains in the brain; a view favoring the modular paradigm in which different brain regions serve as independent processors of different informational aspects (Bressler and Menon, 2010). An alternative view emphasizes the co-operation between different brain regions in large-scale neural networks. Higher order mental processes, such as attention, executive functioning, language and memory, cannot be localized to a single brain region. Instead, these functions are subserved by neural networks spanning across different cortical regions (Mesulam, 1990). This view focuses on how different brain systems interact through functional and structural connectivity.

Functional connectivity in the brain

Functional organization in the brain follows two principles: *integration* and *segregation*. Segregation refers to the existence of specialized neurons or brain areas that are organized into neuronal populations which, in turn, form functionally specialized cortical regions. Integration gives rise to the coordinated activity of these neuronal populations, mediated by functional connectivity (Friston, 1994). Functional connectivity may be defined as the temporal correlation of two spatially remote neurophysiological events (Friston et al., 1993), and may be assessed through neuroimaging techniques, such as fMRI. The interplay between segregation and integration in neural networks facilitates the complex cortical information processing that underlies cognitive functions (Friston, 1994).

Structural connectivity in the brain

Cortico-cortical connections

The neocortex is the part of the brain responsible for most higher mental activities, and receives inputs from and transmits information to other cortical regions, subcortical structures and the thalamus. Cortical neurons are organized into layers and each layer contains different inputs and outputs. Most of the neocortex is arranged in six distinctive layers, with each layer containing different cell types and dendrites of specific cortical neurons. Layer I, the molecular layer, contains very few neurons; layer II is the external granular layer; layer III the external pyramidal layer; layer IV the internal granular layer; layer V the internal pyramidal layer; and layer VI is the multiform or fusiform layer. Layer IV primarily receives thalamocortical connections while layers V and VI connect the cerebral cortex with subcortical regions. Layer V gives rise to all of the principal cortical efferent projections to basal ganglia, brain stem and spinal cord. Layer VI, the multiform or fusiform layer, projects primarily to the thalamus (Shipp, 2017).

Layers II and III, also known as the supragranular layers, are the primary origin and termination of intracortical connections, which are either associational (i.e., with other areas of the same hemisphere); or commissural (i.e., connections to the opposite hemisphere, primarily through the corpus callosum). Layer III is called the external pyramidal cell layer and may be subdivided into a superficial and a deeper level. Neurons located in the deeper level are larger than the neurons in more superficial levels. Pyramidal neurons in layer III have local projections to neurons in the same cortical area as well as to other cortical areas.

Additionally, they receive intrinsic and feed-forward excitatory inputs from other cortical areas as well as excitatory thalamic input (Shipp, 2017). Layer III thus serves as the major site for cortico-cortical and thalamo-cortical integration and plays a critical role in information processing (Vogt and Pandya, 1978).

Pyramidal neurons

The neocortex essentially contains two types of neurons: smooth inhibitory neurons in which the dendrites are without spines, and excitatory stellate and pyramidal neurons whose dendrites bear spines. Dendritic spines are small membranous actin-rich protrusions serving as the principal site of excitatory inputs to glutamatergic neurons (Hering and Sheng, 2001).

Pyramidal neurons constitute approximately two-thirds of cortical neurons and are located in all cortical layers except layer I, the molecular layer. Their most prominent feature is an apical dendrite that may extend through all layers toward the pial surface, and multiple basilar dendrites that extend laterally. Pyramidal neurons may be subcategorized on the basis of their morphology and functional features, and each cortical layer may contain pyramidal neurons that are exclusive to that layer (Shepherd, 2004).

Dendritic spines

Neurons communicate by means of synaptic transmission at the *synapse.* Approximately 95% of excitatory synapses on cerebral pyramidal cells occur on dendritic spines, and each spine typically receives one synaptic input (Arellano et al., 2007).

Spines are composed of specialized subdomains that exert different functions in synaptic transmission (Bosch and Hayashi, 2012). Located on the membrane on the tip of the spine is the post-synaptic density (PSD), a specialized site consisting of a multitude of different proteins including receptors, scaffolding proteins and ion channels (Verpelli et al., 2012). Spines are typically club shaped with a head of approximately 1 μm and a shaft of about 0.1 μm diameters (Harris and Kater, 1994); however, they may take on many shapes and sizes. The most commonly used nomenclature divides spines into three classes based on the appearance and size of their head and neck; the stubby spine, the thin spine and the mushroom spine (Nimchinsky et al., 2002). The density of spines on mature dendrites is typically 1 to 10 spines per μm (Sorra and Harris, 2000); however, regional differences in spine density are documented (Benavides-Piccione et al., 2002). Elston (2000) found that dendrites in frontal areas had higher spine densities than dendrites in temporal, parietal and

occipital lobes, in primates. This is likely to reflect variations in function and connectivity within the different regions.

The different shapes of spines are suggestive of their function as the postsynaptic compartment isolating biochemical signals, such as calcium (Koch and Zador, 1993). These signals are essential for the induction of synaptic plasticity (Nimchinsky et al., 2002, Tønnesen et al., 2014). Synaptic plasticity refers to the ability of synapses to modify their strength in response to fluctuations in their activity (Citri and Malenka, 2008). Some studies suggest that the shape and size of the PSD may be altered by changes in synaptic activity (Desmond and Levy, 1986, Buchs and Muller, 1996). Studies have found a positive correlation between the size of the spine and the PSD, the number of receptors and synaptic strength (Sala et al., 2001), strongly suggesting that spine structure reflects synaptic function. Several lines of research have also provided strong evidence that neuronal activity may modify spine morphology, and morphological changes have been associated with long-term synaptic plasticity (Yuste and Bonhoeffer, 2001, Harris et al., 2003).

The exact purpose of dendritic spines is unclear, although it has become evident that they play an important part in brain connectivity and synaptic plasticity (Hering and Sheng, 2001). The ideas regarding the function of spines can generally be grouped into three somewhat different proposals; spines may enhance synaptic connectivity; spines may modify synaptic potentials; and third: spines may implement input-specific synaptic plasticity (Stapanyants et al., 2002). It has been argued that these suggestions reflect the same basic function: to form neural circuits that are distributed across the entire brain and are plastic in nature (Yuste, 2011). Importantly, dendritic spines are considered to be the basic units subserving neuronal integration (Yuste and Denk, 1995), and are therefore closely related to functional connectivity.

Abnormal anatomical connectivity in schizophrenia: evidence from neuropathology

In a pioneer study using computed tomographic (CT) scans, Johnstone et al. (1976) reported dilated cerebral ventricles in a group of patients diagnosed with schizophrenia. Since then, and with the advent of magnetic resonance imaging (MRI), enlargement of the ventricles has become an established anatomical finding in schizophrenia confirmed by several studies and meta-analyses (Weinberger et al., 1979, Pakkenberg, 1987). In line with this finding, reduced gray matter, also referred to as cortical thinning, has also been observed in schizophrenia (Zipursky et al., 1992, Harvey et al., 1993). These brain anomalies have been demonstrated in both first-episode (Steen et al., 2006), and chronic patients (Ellison-Wright et al., 2008), as well as at-risk individuals (Witthaus et al., 2009, Brent et al., 2013).

A reduction in cortical thickness could theoretically be due to cell atrophy. However, although there are some evidence indicating that the brain alterations associated with schizophrenia may be progressive in nature (Kempton et al., 2010), it is generally accepted that schizophrenia is not a degenerative disease (Rund, 2009). Studies have failed to show any difference in the total cortical cell number between healthy subjects and patients with schizophrenia (Pakkenberg, 1993). Furthermore, absence of gliosis is another common histological finding (Harrison, 1999). Gliosis, or scar tissue development in the brain, would be expected to occur if schizophrenia was indeed degenerative in nature. These findings indicate that cortical thinning is due to other factors than neuronal loss.

Along with the reduction in gray matter in some regions of the brain in schizophrenia, morphological post mortem studies have also demonstrated an increase in neuronal density. That is, the interneuronal space or neuropil is reduced (Selemon et al., 1995, Selemon et al., 1998, Buxhoeveden et al., 2000). Selemon et al. revealed abnormally high neuronal density coupled with decreased volume in Brodmann areas 9 (1995), and 46 (1998), which are areas corresponding to the dorsolateral prefrontal cortex (DLPFC). In area 9, neuronal density was 17% higher in the brains of schizophrenic patients compared to healthy subjects, while in area 46 the corresponding number was 21% ($p = .016$). Other studies have also demonstrated changes in neuronal and glial somal size in the PFC in schizophrenia (Rajkowska et al., 1998).

Neuropil is mainly composed of dendrites; dendritic spines; and axon terminals, and thus serves as the major location for synapses (Somenarian, 2012). Alterations in neuropil are therefore suggestive of synaptic and dendritic reorganization. This finding has spurred the

reduced neuropil hypothesis (Selemon and Goldman-Rakic, 1999), which postulates that the neuropathology in schizophrenia entails changes in cellular architecture affecting neuronal processes, which in turn compromise brain circuitry and cortical information processing.

Dendritic spine pathology

Recent enquiry has provided evidence that structural alterations of spiny synapses might be relevant for the pathology in several neuropsychiatric diseases (Penzes et al., 2011, Lai and Ip, 2013). Serving as the principal site for synaptic input, spines play a crucial role in brain connectivity and plasticity. A common characteristic of many neuropsychiatric diseases, including schizophrenia, is that of severe disruptions in higher executive functions and information processing, functions that heavily rely on the communication between different brain regions. Spine pathology may be subdivided into two categories: altered distribution and altered ultrastructure. The former may include altered spine numbers, spine shape, and location on the dendrite, while the latter refers to changes affecting cellular organelles within the spine (Fiala et al., 2002).

Consistent with the neuropil hypothesis (Selemon and Goldman-Rakic, 1999), which postulates that cortical thinning might be caused by a reduction of neuronal processes; a few studies have found that dendritic spines might be the site for changes in schizophrenia. A consistent finding in some areas in the schizophrenic brain is that of reduced spine density on pyramidal neurons, especially in the prefrontal areas. Garey et al. (1998) investigated dendritic spine density on pyramidal neurons in layer III of frontal and temporal cortex of schizophrenic patients, and found a significant reduction. The numerical spine density in temporal cortex was 0.27/µm in the healthy control group and 0.11/µm in schizophrenia. The corresponding numbers in frontal cortex were 0.30/µm and 0.10/µm, respectively. In a study by Glantz and Lewis (2000), spine densities on basilar dendrites of pyramidal neurons in layer III of the DLPFC (area 46) and primary visual cortex (area 17) in schizophrenia were compared with those of patients with other psychiatric diseases and healthy controls. Mean spine density in deep layer III of the DLPFC in schizophrenic subjects was 23% lower than that of healthy controls (figure 1.1) and 16% lower compared to the psychiatric subjects. No significant difference was found in superficial layer III between groups. Additionally, no significant differences in spine density between groups were observed in area 17. These

studies imply that reduced spine density in schizophrenia might be region- and layer specific, and furthermore, that DLPFC is a core site of dysfunction in schizophrenia.

Alterations in spine density and morphology indicate changes in synaptic transmission and connectivity, a view that is further corroborated by immunocytochemical findings. Presynaptic proteins may serve as markers of synaptic input to neurons, and changes in expression may correlate with disturbances in synaptic transmission. Glantz and Lewis (1997) found decreased synaptophysin immunoreactivity in the DLPFC in subjects with schizophrenia compared to matched healthy controls, suggestive of a decrease in the number of presynaptic terminals. A loss of presynaptic input is consistent with a decrease in dendritic spine density on postsynaptic neurons. Alterations in synaptophysin expression might not be uniform across brain areas. In the cingulate cortex, increased expression has been reported (Gabriel et al., 1997). This could indicate that the pattern of neuronal pathology in schizophrenia differs across brain regions.

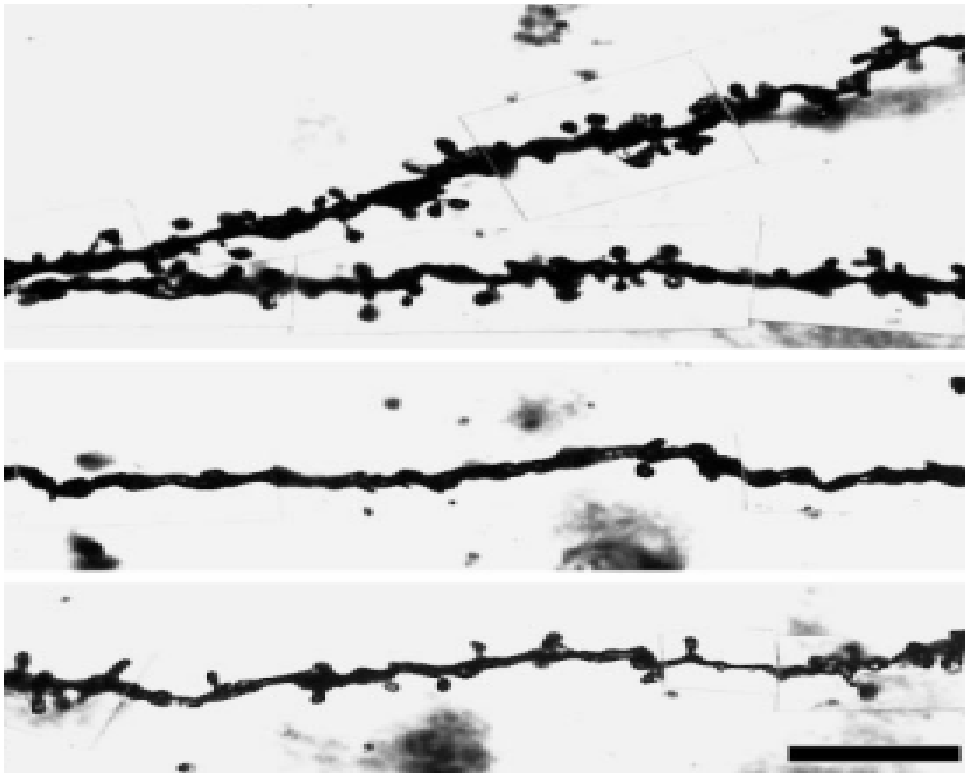

Figure 1.1 Brightfield microscope image showing Golgi-impregnated basilar dendrites and spines on layer III pyramidal neurons in DLPFC. The uppermost image is from a healthy control subject, while the lowermost images are from two patients with schizophrenia. The scale bar is set to 10 μm (Glantz and Lewis, 2000).

Aberrant neurodevelopment in schizophrenia

Abnormal synaptic reorganization in schizophrenia is a reliable finding. However, there is little consensus in regards to the timing of these events. Neuropathological studies on post mortem tissue do not offer insight into when synaptic deficits might have emerged. Establishing the timing of anatomical brain alterations has great implications for the etiology of the disease. During the last decades, the neurodevelopmental model has been one of the prevailing pathogenic hypotheses of schizophrenia. Several lines of enquiry support the model, with the neuropathological data forming a substantial part of the evidence. At its simplest, the model posits that schizophrenia results from aberrant neurodevelopmental processes occurring long before the onset of clinical symptoms, caused by both genetic and environmental factors (Rapoport et al., 2005).

One of the first advocates of the neurodevelopmental model, Weinberger, suggests that events occurring during early childhood and even before birth may interact with neural processes taking place during adolescence or early adulthood to trigger the outbreak of symptoms (Weinberger, 1987). This model is sometimes referred to as the *early* developmental model. An alternative model, which could be referred to as the *late* developmental model, suggests that schizophrenia is a consequence of abnormal maturational processes of the cerebral cortex during postnatal development (Hoftman and Lewis, 2011, Uhlhaas, 2011). Normal postnatal brain development is characterized by an initial overproduction of neuronal processes during the first years of life followed by a selective elimination of synaptic connections (Huttenlocker, 1979). Synaptic pruning is present in most cortical areas; however, with a different time-course. Prefrontal and other association areas are the last to complete synaptic maturation, occurring in the mid to late adolescence, hypothesized to be due to the complexity of these regions (Huttenlocker and Dabholkar, 1997). During adolescence and up until young adulthood the brain undergoes drastic functional and structural reorganization. This is the timing of the onset of schizophrenia in most cases. Feinberg (1982) suggested that schizophrenia involved a disruption in synaptic pruning. Since the initial proposal, several studies have indicated that schizophrenia is a disorder of developmentally reduced synaptic connectivity (McGlashan and Hoffman, 2000, Beneyto and Lewis, 2011), with one of the most compelling evidence being reduced dendritic spine density in prefrontal areas (Garey et al., 1998, Glantz and Lewis, 2000.)

Evidence corroborating the neurodevelopmental model of schizophrenia stems from several lines of enquiry. Firstly, subtle cognitive and behavioral deviations may be observed

years before the onset of illness in individuals who develop schizophrenia (Strous et al., 1994, Cannon et al., 1997, Fuller et al., 2002), which suggests that premorbid functional abnormalities are present from an early age and are aggravated at the time of onset. Secondly, many of the structural abnormalities associated with schizophrenia, such as a subtle gray matter loss and enlargement of the ventricles, appear to be present at onset of disease (Byun et al., 2012, Brent et al., 2013) and studies investigating progression of these abnormalities during the course of illness are inconclusive. Substantial progression of structural abnormalities is to be expected if they are symptomatic of neurodegeneration (Rund, 2009). Some of the studies that have indeed reported progression might include confounding effects of neuroleptic medication or drug use (Fusar-Poli et al., 2013, Van Haren et al., 2013).

Furthermore, molecular evidence from studies investigating the expression of key developmental genes speaks in favor of a neurodevelopmental origin in schizophrenia (Walsh et al., 2008). Notably, many of these genes are also involved in synaptic plasticity (Hall et al., 2015); further supporting the notion that schizophrenia is a disease of the synapse.

Abnormal functional activity and connectivity: evidence from neuroimaging

Hypofrontality

The advent of new technological approaches in neuroscience has enabled research to move beyond structural neuropathology. Great scientific emphasis currently lies on the pathophysiological aspects associated with schizophrenia, and neuroimaging techniques have made it possible to assess functional brain disturbances underlying the clinical symptoms.

In 1974, Ingvar and Frantzen made the seminal discovery of hypofrontality. Using Xenon-enhanced CT scanning on patients with schizophrenia during task performance requiring prefrontal involvement, the authors observed a decline in regional cerebral blood flow (rCBF) in the PFC. rCBF is a measure closely related to neuronal metabolism, and a reduction implies a decrease in neural activity. Since the initial observation, hypofrontality has become a recurrent finding, especially in the DLPFC (Weinberger et al., 1986, Riehemann et al., 2001, Perlstein et al., 2001).

The DLPFC (Brodmann areas 9 and 46) plays a key role in working memory. Working memory is defined as the process whereby transient mental representations form the basis for abstract thought (Baddeley, 1992), or alternatively: the ability to maintain memories temporarily online to permit further cognitive processing (Goldman-Rakic, 1999). The term

thus encompasses information storage and various executive processes, such as attentional control, inhibitory control, planning and updating. Working memory deficits represent an integral part of the cognitive symptoms of schizophrenia. Patients with schizophrenia typically perform poorly on tasks that depend on working memory, such as the delayed response test, stroop test (measuring cognitive inhibition), and the wisconsin card sort test (cognitive flexibility) (Goldman-Rakic and Selemon, 1997). Working memory function has proven to be a strong predictor of long-term outcome (Green et al., 2000). Considerable evidence points towards a functional link between working memory dysfunction and hypoactivity in the DLPFC (Carter et al., 1998, Perlstein et al., 2001).

Functional connectivity

It is widely accepted that the PFC is central to the pathology of schizophrenia, with several studies confirming the abnormal activation pattern in DLPFC during working memory performance (Goldman-Rakic and Selemon, 1997). However, as previously discussed, it is generally acknowledged that brain regions rarely act independently and that cortical information processing is a product of interregional co-operation (Mesulam, 1990). A question thus arises as to whether working memory disturbances can merely be ascribed to dysfunction of the PFC, or if other brain regions are involved.

The latter suggestion has been implicated by several studies (Meyer-Lindenberg et al., 2001, Kim et al., 2003, Frith et al., 2005). During the past three decades, the concept of functional connectivity has gained much interest in the research field of schizophrenia. The evidence for localized brain abnormalities associated with the disease is scarce and subtle, and there is strong agreement that schizophrenia instead involves disrupted integration between functionally specialized regions. This integration is mediated by functional connectivity between different areas (Friston, 1994).

A large number of studies conducted on functional connectivity in schizophrenia use blood-oxygen-level dependent (BOLD) fMRI signaling, assessing task related activity in the brain. An emergent finding from these studies is a significant reduction in functional connectivity in patients with schizophrenia during various cognitive based tasks, including working memory. Henseler et al. (2010) found that patients with schizophrenia showed reduced functional connectivity between the DLPFC and other regions involved in working memory processes, such as the hippocampus. Reduced functional frontotemporal connectivity has previously been reported by other studies (Meyer-Lindenberg et al., 2001,

Frith et al., 2005) and furthermore, reduced functional frontoparietal connectivity is also a finding from fMRI studies (Kim et al., 2003).

Disconnection hypothesis

Neuroimaging studies have provided convincing evidence that schizophrenia is associated with decreased activity within brain regions (i.e., hypofrontality), and decreased functional integration between different areas. Aberrant functional connectivity is consistent with the finding of synaptic reorganization in schizophrenia from neuropathological studies. The DLPFC has been associated with hypofrontality and decreased functional connectivity (Perlstein et al., 2001). Additionally, it is also one of the areas in which dendritic spine density has been shown to be decreased in patient samples (Garey et al., 1998, Glantz and Lewis, 2000), consistent with altered cortical connectivity.

This evidence is in line with the hypothesis proposed by Friston (1998). The disconnection hypothesis suggests that the mechanism underlying the pathology in schizophrenia is a global decrease in structural and functional connectivity affecting an array of cognitive processes. An important assumption of this hypothesis, according to the author, is that a decline in structural connectivity may associate with white matter and gray matter differently. That is, whereas the integrity of white matter connectivity may be maintained, synaptic connections between neurons could be distorted (Friston, 1998).

Nevertheless, while several lines of research support this view, there are some incongruent findings that challenge the disconnection hypothesis. One of them is the relatively new discovery of resting state networks and their contribution in neuropsychiatric diseases, such as schizophrenia.

Resting state networks

The symptoms of schizophrenia include several disabling deficits in cognitive functioning and in recent years, much of the scientific focus has shifted towards the functional contribution of resting state activity on cognition. Resting state activity refers to the intrinsic neural activity observed when subjects are not engaged in an explicit task and may be measured by fMRI (Northoff et al., 2010). Fluctuations in the BOLD signal during wakeful rest are interpreted as reflections of the neural baseline activity in the brain, representing the internal mental state in the absence of goal directed neural activity and

external sensory input. These fluctuations may be detected in the low frequency range and are believed to correspond to resting state networks (Damoiseaux et al., 2006, Heine et al., 2012).

Default mode network in schizophrenia

Activity and functional connectivity

The most widely studied resting state network is the default mode network (DMN). The DMN refers to a distributed set of brain regions that are more active during wakeful rest than during task oriented behavior, and it is characterized by a high degree of internal functional connectivity (Raichle et al., 2001). Supported by fMRI studies, the DMN is believed to mediate stimulus-independent thought and self-referential thinking. The DMN subserves mental exploration that is detached from the external world, is introspective and based on autobiographical memory and thoughts about the future (Buckner et al., 2008). The brain regions most often associated with the DMN are the medial prefrontal cortex (MPFC), posterior cingulate cortex (PCC), lateral parietal lobes and medial temporal structures (Raichle et al., 2001). The MPFC and the PCC are regarded as the main functional hubs in the DMN (Buckner et al., 2009).

Activity in the DMN is negatively correlated (anticorrelated) during rest with activity in the task positive network (TPN) (Fox et al., 2005). This phenomenon has been referred to as task suppression of the DMN. Greater task suppression of the DMN, and greater anticorrelation between the DMN and the TPN, is associated with better performance in goal oriented tasks with high attentional demands. These observations suggest that the brain has essentially two distinct modes of processing, the DMN, which is internally focused, and the TPN, which is externally focused (Vanhaudenhuyse et al., 2011).

The TPN comprises regions that display relatively high activity during task performance that requires attention and working memory capacity. The network is localized in frontal and parietal regions, and includes the DLPFC (Toro et al., 2008). It is therefore also referred to as the frontoparietal network. As previously discussed, schizophrenic subjects consistently show diminished activity in the DLPFC during performance of working memory tasks (Carter et al., 1998, Perlstein et al., 2001).

A number of studies have linked the DMN to several neuropsychiatric disorders, including depression (Dutta et al., 2014) and schizophrenia (Whitfield-Gabrieli and Ford, 2012). Contrary to the majority of fMRI studies on schizophrenia that demonstrate significant

reduction in activity and connectivity, neuroimaging studies have consistently found the DMN to be functionally hyperactive and hyperconnected in schizophrenia (Whitfield-Gabrieli et al., 2009, Mannell et al., 2010, Guo et al., 2015). Guo et al. (2015) investigated DMN functional connectivity in first-episode schizophrenia patients. All patients were medication-naïve, which excluded the possibility of confounding effects of medicine. It is still unclear to what extent antipsychotic drugs may affect brain connectivity; however, they have been shown to confound with fMRI results in schizophrenia (Abbott et al., 2013). The authors divided functional connectivity into either long- or short-range connectivity depending on their anatomical distance and found that both short- and long-range functional connectivity were increased in the anterior and posterior DMN in schizophrenia patients (Guo et al., 2015). Whitfield-Gabrieli et al. (2009) found that schizophrenic patients as well as first-degree relatives exhibited significantly reduced task suppression of the DMN during working memory performance, and during rest and task, patients and their relatives exhibited functional hyperconnectivity of the DMN. Increased functional connectivity within the DMN coincided with decreased anticorrelation between the MPFC and the DLPFC. The authors suggest that reduced task suppression of the DMN could partly account for the working memory deficits schizophrenic patients often exhibit. Falkenberg et al. (2015) found that people at high risk of developing psychosis showed a marked failure to deactivate the MPFC during a working memory task, compared to healthy controls. Reduced anticorrelations between the DMN and the TPN may result in a difficulty with switching between internal and external modes of attention. In patients, the magnitude of functional hyperconnectivity within the DMN is associated with the severity of clinical features (Whitfield-Gabrieli et al., 2009). The disturbance in the DMN in relatives lends support to the notion of a strong genetic component in the etiology of schizophrenia. A study from 2013 (Tang et al.) also reported increased functional connectivity between the MPFC and PCC in early-onset cases of schizophrenia (onset before the age of 18), also providing support in the direction of a neurodevelopmental account with genetic implication.

In addition to offering an explanation for the cognitive challenges that schizophrenic patients experience, abnormal DMN activity in schizophrenia may also account for many of the cardinal psychotic symptoms of the disease. Hyperactivation of the DMN may disrupt the boundary between internal thought and external percepts, possibly giving rise to hallucinations, thought insertion and delusions of control (Robinson et al., 2016). The DMN facilitates self-referential thinking, and a hyperactive DMN could promote an exaggerated

focus on one's own thoughts and emotions that could possibly lead to an overwhelming sense of self-relevance in the external world. This could explain paranoid delusions in which the patient may believe that individuals or groups are conspiring against her. Additionally, the degree of functional hyperconnectivity between different regions within the DMN is positively correlated with the global scale for the assessment of positive symptoms summary score (Whitfield-Gabrieli, 2009) as well as the positive and negative syndrome scale (Tang et al., 2013).

Structural connectivity

Resting state networks, including the DMN, are functional networks approached through fMRI. While fMRI studies may reveal functional connectivity between brain regions, they cannot elucidate how, or if, these brain regions are structurally connected. Consequently, although the DMN is an anatomically defined network and the brain regions involved have been identified, the structural connectivity within the network is less understood. In an attempt to identify structural connectivity in the DMN, Greicius et al. (2009) used DTI to investigate white matter tracts between the nodes of the network. They hypothesized that the high functional connectivity between the functional hubs in the DMN would be reflected in high structural connectivity, and predicted that direct connections between the MPFC and the PCC would be detectable. The results of the study demonstrated that DMN functional connectivity was, by and large, reflected in a corresponding structural connectivity. These findings were further supported in a study by Horn et al. (2014), using similar technical approach. Combining resting state fMRI and DTI, the authors found strong function-structure agreement in the DMN.

The relation between structural and functional connectivity in the DMN in schizophrenia

The DMN is a resting state network distributed across different brain regions that show high functional connectivity in healthy subjects. A growing body of evidence also suggests that the high functional connectivity is supported by strong structural connectivity between the main nodes of the network (Greicius et al., 2009, Horn et al., 2014). The majority of these studies are conducted on healthy subjects; however, they have great clinical relevance. Compared to healthy controls, patients with schizophrenia demonstrate abnormally

increased functional connectivity in the DMN (Whitfield-Gabrieli et al., 2009, Mannell et al., 2010, Guo et al., 2015); however, the relation between pathology and structural connectivity within the network remains elusive. Assuming strong function-structure agreement, it is plausible that abnormal functional connectivity would be rooted in aberrant structural connectivity.

The majority of studies investigating structural connectivity within the DMN have used *in vivo* imaging techniques, such as DTI, focusing on white matter pathways. Few studies have investigated the relationship between functional connectivity and anatomical structure using gray matter. This is surprising as fMRI is generally interpreted as an indirect measure of neuronal activity, and gray matter is mostly composed of neuronal cell bodies and neuropil. Dendritic spines are believed to be the basic functional units that subserve neuronal integration (Yuste and Denk, 1995). Functional connectivity is an fMRI derived measure of neuronal integration, and is therefore very likely to reflect synaptic communication. Aberrant structural connectivity in other networks in schizophrenia is well documented, albeit is hypothesized to originate from abnormalities in gray matter rather than white matter. Several studies have reported synaptic reorganization in pyramidal neurons in schizophrenia, affecting e.g., the density of dendritic spines, believed to contribute to aberrant wiring (Selemon et al., 1995, Garey et al., 1998, Glantz and Lewis, 2000). This could imply that if the abnormal functional pattern of DMN connectivity in schizophrenia has a structural basis, it is likely to originate from gray matter anomalies. Studies investigating gray matter connectivity in the DMN are, however, scarce and the synaptic correlate of increased functional connectivity in schizophrenia remains uncertain. Vidal-Pineiro et al. (2014) investigated the effect of aging on functional connectivity between the MPFC and the PCC in healthy subjects. The DMN has previously been shown to be highly susceptible to the effects of aging (Damoiseaux et al., 2008). Vidal-Pineiro et al. (2014) found that functional connectivity within the network decreased as a function of age and, most importantly, a decline in functional connectivity correlated with a loss of gray matter integrity. This finding suggests that functional connectivity in the DMN is not only reflected in white matter connectivity, but also in synaptic connectivity.

Aberrant brain connectivity in autism spectrum disorder: implications for schizophrenia

Schizophrenia and autism spectrum disorder (ASD) are both neuropsychiatric diseases with high heritability. Although they differ on several parameters, such as clinical presentation and onset of symptoms, they share some common mechanisms in regards to developmental neurobiology (Waltereit et al., 2014) as well as cognitive functioning (Goldstein et al., 2002). In fact, prior to the DSM-III, ASD was often referred to as childhood schizophrenia. Findings from genome wide association studies (GWAS) have indicated that ASD and schizophrenia may share the same genetic mutations coding for neurodevelopment and synaptic plasticity (Guilmatre et al., 2009). These findings imply that schizophrenia and ASD may overlap in terms of pathophysiological mechanisms.

As discussed in previous sections, schizophrenia involves aberrant synaptic connectivity. An established finding in the DLPFC is decreased dendritic spine density on pyramidal neurons, correlating with dysfunctional interregional integration in the networks subserving working memory. One of the proposed mechanisms by which decreased spine density might occur is excessive synaptic pruning during postnatal neurodevelopment (Feinberg, 1982). ASD has in similar manners been associated with developmental alterations of excitatory synapses, and abnormal connectivity within and between neural circuits. However, while schizophrenia is most often associated with hypoconnectivity and hypofunction, altered connectivity in ASD is seemingly more complex. An in-depth review of ASD research is beyond the scope of this thesis; however, the neurobiology of ASD could have important implications for aberrant connectivity in schizophrenia. Studies have found evidence for both functional hyper- and hypoconnectivity (Just et al., 2007, Superkar et al., 2013, Uddin et al., 2013) and in contrast to the reduced spine expression typically observed in schizophrenia, ASD is associated with increased spine densities in some brain regions (Hutsler & Zhang, 2010). Increased spine density is a less common outcome of disease than a decrease; however, it is known to occur and may represent a failure in synapse elimination during development. In a study conducted on postmortem ASD temporal lobes, Tang et al. (2014) found increased dendritic spine density on pyramidal neurons in layer V. The spine alterations were associated with a defect in postnatal spine pruning, which correlated with overactive mTOR signaling and impaired autophagy. The authors hypothesized that autophagy modulates synapse maturation downstream of mTOR and that hyperactive mTOR signaling contributes to autophagy deficiency, which in turn leads to the overexpression of

spines as observed in ASD. Hutsler and Zhang (2010) found that increased spine density in layer II of frontal, temporal and parietal regions, as well as layer V in temporal areas, was associated with reduced brain weight, and was most commonly found in patients with ASD who had low levels of cognitive functioning. These findings provide structural support to the suggestions put forward by Markram and Markram (2010). They propose that local neocortical microcircuits in the brains of ASD patients might be hyperfunctional, characterized by hyperplasticity and hyperreactivity. This hyperfunction is likely to lead to connectional changes and, subsequently, altered cortical information processing. Functional hyperconnectivity has been observed across long- and short-range connections in ASD (Kleinhals et al., 2008, Cerliani et al., 2015) and may predict the severity of symptoms. Children with greater functional connectivity have been found to exhibit more severe social deficits (Superkar et al., 2013).

The implication to be made from these observations is that hyperactivity and functional hyperconnectivity, as observed in the DMN of patients with schizophrenia, may possibly be associated with a different anatomical pattern than that of hypoconnectivity. Although dendritic pathology usually associates with a loss of spines, an increase in spine density could also be evident of abnormal synaptic transmission and could also contribute to aberrant connectivity. This suggestion is in line with the aberrant connectivity hypothesis proposed by Gaspar et al. (2009). They argue that the disconnection hypothesis might be obsolete, and that the concept of abnormal communication in the schizophrenic brain might entail both *decreased* and *increased* connectivity within and between brain regions. The authors claim that connectivity in schizophrenia is *disbalanced*, with some areas displaying reduced function and connectivity and others excessive function and connectivity, perhaps as compensatory mechanisms. Assuming that function is indeed a reflection of structure, one could possibly expect the structural basis associated with hyperactivity/connectivity to differ from that of hypoactivity/connectivity. Whereas functional hypoconnectivity in schizophrenia is correlated with a loss of dendritic spines, potential alterations in synaptic connectivity associated with increased functional connectivity is less understood.

The Golgi silver impregnation technique

One common method for investigating synaptic connectivity is by examining the expression of dendritic spines on individual neurons from post mortem tissue (Mancuso et al., 2013). Spines are believed to be the structural support that enables excitatory neuronal

transmission, and the density of spines may serve as a measure of connectivity at a synaptic level.

The Golgi silver impregnation technique is a histological procedure that reveals complete neuronal morphology in light or electron microscopy by staining neurons black against a neutral background. Discovered in 1873 by Camillo Golgi, it has since become a standard technique for investigating dendritic spine density. The procedure consists of two steps: chromation and impregnation. Successful staining depends on the formation of intracellular deposits of silver chromate achieved through the reaction between potassium dichromate and silver nitrate (Angulo et al., 1994, Friedland et al., 2006, Rosoklija et al., 2003). The method will only stain approximately 5% of the neuronal population (Pasternak and Woolsey, 1975), allowing for more precise visualization of individual neurons.

Although the Golgi technique excels at characterization of neuronal morphology, it is also known for its many challenges. The staining is often capricious, time-consuming and dependent on several different aspects of the tissue at hand, with fixation time perhaps being the most important determinant of successful staining. The interval been death and autopsy (post mortem interval) may also affect the outcome of the staining. Because of the challenges fixation time and post mortem interval (PMI) pose on successful Golgi staining, several revisions have been developed over the years.

The Golgi-Kopsch variation (occasionally referred to as Colonnier-Golgi or aldehyde Golgi) is developed to better stain tissue with prolonged fixation time (Colonnier, 1964). It does so by utilizing paraformaldehyde, a commonly used fixative, in the actual staining procedure. The Golgi-Kopsch method has successfully been applied in studies conducted on tissue that has been formalin-fixed for many years (Rosoklija et al., 2003). Although extended fixation time poses a challenge for the Golgi technique, it has not been noted to affect the quality of the staining once it has been achieved (Rosoklija, 2003).

The rapid Golgi staining technique is considered to work best with fresh tissue or tissue that has been fixated for a short period of time (from hours to a few days). Instead of paraformaldehyde, the procedure requires osmium tetroxide in addition to potassium dichromate (Rosoklija et al., 2003).

Lastly, in the Golgi-Cox method, mercuric chloride is used. This variation is reported to be less sensitive to post mortem fixation delay (Buell, 1982).

Aims and hypothesis

The main objective of this thesis was to investigate structural connectivity at the synaptic level in the DMN in schizophrenia.

In order to reach the overall aim, several sub-aims were set; (I) investigate dendritic spine density on layer III pyramidal neurons in the MPFC and the PCC from brain tissue of patients with schizophrenia and healthy control subjects. I hypothesized that spine density would be increased in the schizophrenia subjects compared to the healthy controls, as a reflection of the increased functional connectivity that has been observed in the DMN in schizophrenia in previous fMRI studies. (II) Evaluate regional differences in spine density between the MPFC and the PCC within the schizophrenia sample. Several studies have highlighted the role of the PFC in the pathology of schizophrenia, and I therefore hypothesized that the MPFC would display more severe abnormalities than the PCC, manifested as a higher mean spine density. (III) Investigate the possible effect of age on dendritic spine density. The DMN has been shown to be especially susceptible to age-related changes, and I therefore hypothesized that older subjects would display decreased spine density compared to the younger subjects.

Materials and Methods

Samples

The present study was conducted on formalin-fixed post mortem brain tissue from eight diagnosed schizophrenic medication-naïve patients admitted at the Dikemark psychiatric hospital in the period 1930-1945, provided by Ole A. Andreassen at Oslo University Hospital. The diagnostic criteria in the thirties/forties might have differed somewhat from the DSM and the International classification of diseases (ICD), and so diagnoses have recently been confirmed by psychiatrist and former chief attending physician at Dikemark, Kjell Martin Moksnes. All the patients included in the study met DSM-V criteria for schizophrenia, based upon medical records provided by the Oslo City Archives. The structural abnormalities associated with schizophrenia are believed to be characteristic of the disease as a whole, rather than being restricted to any subtype. Consequently, the present study has not considered the subtypes of individual patients in the sample. Brain tissue was obtained from the left hemisphere, in accord with similar studies. Brain material for the control group was provided by the Division of Anatomy at the Medical Faculty, University of Oslo. The sample included tissue from seven subjects not diagnosed with any severe neurological, medical or psychiatric conditions. Case information for both groups is presented in table 1.1.

Ethical considerations

The present study was ethically approved by The Regional Committee for Medical and Health Research Ethics (REK Sør-Øst) (see appendix 1). The data from each subject was treated anonymously, and may only be identified through case ID number. Identity of each subject was kept confidential.

Case ID	*Diagnosis*	*Age*	*BA*	*R/L*
3647	Schizophrenia	55	10, 23	L
3739	Schizophrenia	67	10, 23	L
2837	Schizophrenia	46	10, 23	L
1827	Schizophrenia	33	10, 23	L
2923	Schizophrenia	30	10, 23	L
3329	Schizophrenia	76	10, 23	L
3301	Schizophrenia	58	10, 23	L
2097	Schizophrenia	37	10, 23	L
01	Control	-	10, 23	L
02	Control	-	10, 23	L
03	Control	-	10, 23	L
04	Control	-	10, 23	L
05	Control	-	10, 23	L
06	Control	-	10, 23	L
07	Control	-	10, 23	L

Table 1.1 Case information for test and control group. BA = Brodmann areas, R/L = right or left hemisphere. Age was not available for the control group.

Regions of interest

The regions of interest were delineated according to Brodmann's cortical areas (Brodmann and Garey, 1994). This is the most commonly used nomenclature when identifying the regions involved in the DMN. The MPFC corresponds to Brodmann area 10 and 32, while the PCC corresponds to Brodmann area 23 and 31. The present study collected tissue from area 10 and 23 (figure 2.1).

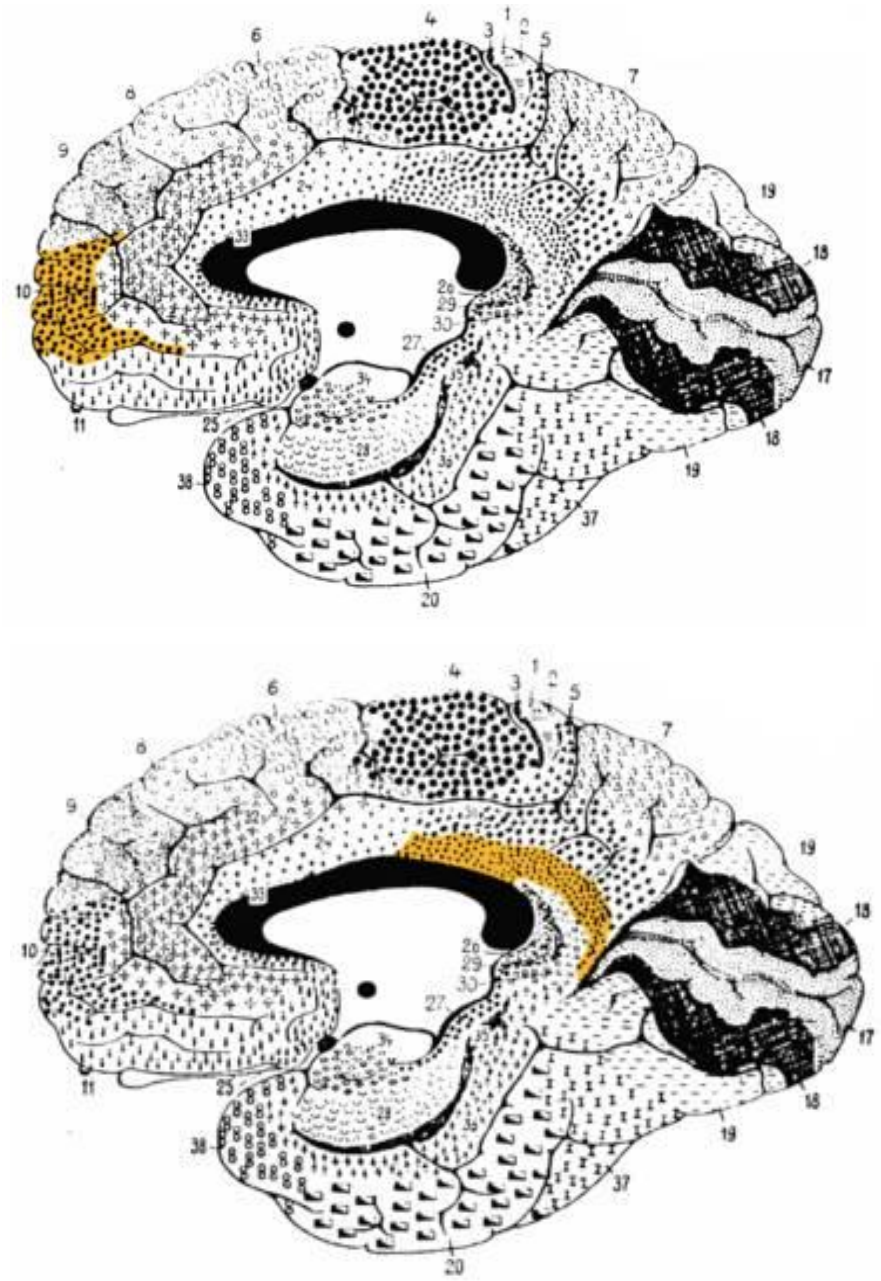

Figure 2.1 Delineation of regions of interest according to the classification by Brodmann (Brodmann and Garey, 1994). The uppermost image highlights area 10 in the MPFC. Area 23 (PCC) is highlighted in the lowermost image.

Preparation and staining of brain tissue

Brain tissue was processed with the Golgi silver impregnation technique. Several revisions of the technique have been developed in order to deal with the challenges of prolonged fixation time. Nevertheless, successful Golgi staining requires that the protocol is optimized to fit the tissue being treated. A substantial part of the current project has thus been allocated to experiments with, and modifications of, different Golgi staining techniques. The control brain tissue included in the study has been fixated for merely 11-12 months, while the schizophrenia brain tissue has been fixated for approximately 7 decades. Test and control groups were therefore exposed to different revisions of the Golgi technique, with varying degrees of success.

The method applied to the schizophrenia specimens was adopted from Rosoklija et al. (2003), who successfully stained human brains that had been fixated for up to 50 years with the use of a Golgi-Kopsch variation. Although this method did fully stain several neurons in the present study, the staining reagents did not penetrate the tissue adequately. Consequently, stained neurons were only observable in the superficial regions of the tissue blocks. In order to detect cortical layer III, a few adjustments were carried out. These experimental conditions included the introduction of Triton-X 100 to the chromation solution to promote better cell permeability (Tokuno et al., 1990), different temperatures during incubation (Ranjan and Mallick, 2010), and brushing off precipitates from the surface of the tissue blocks every 24 hours during chromation (Friedland et al., 2006). Nevertheless, none of these procedures improved the quality or quantity of the staining.

Although the exact chemical mechanisms involved in the Golgi technique remain uncertain, some authors have emphasized the importance of the pH-value of the chromation solution for penetrating the tissue properly (Angulo et al., 1994). The pH serves as an indicator of the reduction of hexavalent chromium to trivalent chromium, which is a crucial step in the chromation process. Over a period of 8 to 12 hours into incubation, the pH of the solution should ideally increase from ~4.0 to ~4.7. This value represents the end of successful chromation, and at this point, the solution should be replaced with freshly made chromation solution. Rosoklija et al. (2003) obtained optimal results when chromation fluids were changed every 12 hours. The present study made some adjustments to the starting pH-value, inspired by the observations made by Bertram and Ihrig (1956). They reported superior penetration and staining when the Golgi method was carried out at even lower pH, and suggested that chromation at pH 3.1 led to optimal impregnation. In the current study,

improved staining of neuronal processes was obtained when the starting pH-value of the chromation solution was set to 3.1, instead of 4.0. Furthermore, the reagents reached deeper levels of the tissue, including layer III. A full description of the staining procedure is provided in table 2.1.

For the treatment of the control brain tissue, both the Golgi-Cox technique and the rapid Golgi technique were tested. The protocol for the rapid Golgi technique was also adopted from Rosoklija et al. (2003) and resembles that of the Golgi-Kopsch variation. However, instead of paraformaldehyde, osmium tetroxide is used in the chromation step and the protocol does not require several changes of chromation solution. A description of the staining procedure is provided in table 2.2.

For the Golgi-Cox procedure, the present study used the FD Rapid GolgiStain™ Kit from FD NeuroTechnologies Inc. The kit provides all the necessary reagents and equipment needed, as well as a protocol. The whole staining procedure is described in table 2.3.

Imaging and quantification

From each region of interest, 10 layer III pyramidal neurons were selected for imaging and subsequent analysis, making up a total of 80 neurons. A dendritic segment of 50 μm from each neuron was imaged in a Z-stack with X100 oil immersion objective using Leica Brightfield microscopy (DM5500B). The dendritic segment was initialized 75 μm from the soma. According to Benavides-Piccione et al. (2002), this is the location on the dendrite in which spine density is highest. Cortical layers were identified by counting layers from the surface, according to the description by Brodmann (Brodmann and Garey, 1994). Layer III was chosen due to its involvement in cortico-cortical connectivity (Vogt and Pandya, 1978) and because previous studies have reported synaptic alterations in cells located in this layer (Glantz and Lewis, 2000).

Criteria for the selection of neurons were adopted from Glantz and Lewis (2000) and included the following:

- Cell soma was located in layer III.
- Full impregnation throughout the neuron, including dendritic tree.
- Neither soma nor the dendrites were obscured by overlying artifacts.
- Presence of at least 3 basilar dendrites, of which one was chosen for imaging.

A few modifications to the Z-stack images were carried out using the microscopy software (Leica Application Suite X), enabling subsequent processing in image processing program ImageJ. Colors were inverted and the brightness and intensity were adjusted. Dendrites were semi-automatically reconstructed using NeuronStudio. The Z-stack images allow for the construction of a maximum intensity projection that resembles the 3D structure of the dendrites. Spines were subsequently detected and counted. The spine density was defined as the number of spines per μm.

Statistical analysis

Statistical analyses were performed with IBM SPSS Statistics 22 software. Assumptions for the parametric techniques were tested, including normal distribution and independence of observations. Shapiro Wilk's test for normality confirmed normal distribution of scores, further supported by inspection of histogram and normal QQ-plot. Each dendrite was considered to be independent within the same brain. The potential threat of outliers was assessed by looking at the difference between the mean and the 5% trimmed mean.

An important aim of the present study was to test for significant differences in dendritic spine density in the DMN between healthy control subjects and subjects with schizophrenia, as well as significant differences in spine density between the two main regions of the DMN. In both cases, two independent groups would be compared and measured on one dependent variable (dendritic spine density). Thus, the independent samples T-test would be applied to the data. Assumptions for the T-test would be tested, including Shapiro-Wilk's test for normal distribution and Levene's test for homogeneity of variances.

Additionally, a possible effect of age on spine density was investigated. Each subject in the group was treated as one independent age group. Thus, more than two independent groups were compared. The assumptions for analysis of variance (ANOVA) were tested, including normality of distribution (Shapiro-Wilk's) and equality of variance (Levene's test), and ANOVA was applied to the data. If the effect of the independent variable (age) yielded a significant effect, post hoc comparisons using the Bonferroni procedure were conducted in order to determine which of the age groups differed significantly.

STEP	*DESCRIPTION*
I Chromation	Pieces of brain tissue were trimmed to 1.5 cm^2 by 3 mm thickness, wrapped in gauze and incubated in 100 ml of a PBS solution containing 5.5% paraformaldehyde and 4% potassium dichromate, in room temperature and in the dark, on a shaker, for 96 hours. pH was monitored periodically. When pH had increased from 3.1 to 3.8, the exhausted solution was replaced with freshly made chromium solution. This corresponded to approximately every 12 hours.
II Silver nitrate	Tissue blocks were washed in increasing concentrations of silver nitrate (0.25%, 0.50%, 0.75%, 1%), 5 minutes each. Tissue blocks were then shaken in 1% silver nitrate in the dark for 1 week.
III Dehydration	Tissue blocks were then washed in ethanol/acetone (1:1) for 2 hours and then ethanol for 5 minutes.
IV Sectioning and mounting	Tissue blocks were sectioned at 90 μm using a vibratome (Leica VT 1000S) and mounted on microscope slides with sufficient amounts of Eukitt® quick hardening mounting medium and cover slipped.
V Imaging	From each region of interest, 10 layer III pyramidal neurons were selected for imaging and analysis. From each neuron, a segment of 50 μm from a basilar dendrite was imaged in a z-stack (Leica Brightfield microscopy, x100 objective). Colors were inverted and intensity and brightness adjusted.
VI Reconstruction and quantification	Dendrites were reconstructed semi-automatically using image processing program NeuronStudio, with some manual adjustments. Spines were subsequently detected and counted.
VII Statistical analysis	Appropriate statistical measures were applied to the data.

Table 2.1 Description of the Golgi-Kopsch method, adopted from Rosoklija et al. (2003), with adjustments.

STEP	*DESCRIPTION*
I Chromation	Pieces of brain tissue were trimmed to 1.5 cm^2 by 3 mm thickness, wrapped in gauze and incubated in 100 ml of a PBS solution containing 2% potassium dichromate and 0.16% osmium tetroxide, in room temperature and in the dark, on a shaker, for 1 week.
II Silver nitrate	Tissue blocks were washed in increasing concentrations of silver nitrate (0.25%, 0.50%, 0.75%, 1%), 5 minutes each. Tissue blocks were then shaken in 1% silver nitrate in the dark for 1 week.
III Dehydration	Tissue blocks were then washed in ethanol/acetone (1:1) for 2 hours and then ethanol for 5 minutes.
IV Sectioning and mounting	Tissue blocks were sectioned at 90 μm using a vibratome (Leica VT 1000S) and mounted on microscope slides with sufficient amounts of Eukitt® quick hardening mounting medium and cover slipped.
V Imaging	From each region of interest, 10 layer III pyramidal neurons were selected for imaging and analysis. From each neuron, a segment of 50 μm from a basilar dendrite was imaged in a z-stack (Leica Brightfield microscopy, x100 objective). Colors were inverted and intensity and brightness adjusted.
VI Reconstruction and quantification	Dendrites were reconstructed semi-automatically using image processing program NeuronStudio, with some manual adjustments. Spines were subsequently detected and counted.
VII Statistical analysis	Appropriate statistical measures were applied to the data.

Table 2.2 Description of the rapid Golgi method adopted from Rosoklija et al. (2003).

STEP	*DESCRIPTION*
I Tissue preparation	Pieces of brain tissue were trimmed to 1.5 cm^2 by 3 mm thickness and immersed in 20 ml of a mixture made from equal volumes of Solution A and B*, and stored in room temperature for 2 weeks in the dark, on a shaker. The mixture was replaced after 24 hours. Tissue was transferred to Solution C** and stored in room temperature in the dark for 1 week. The solution was replaced after 24 hours.
II Sectioning and Mounting	Tissue blocks were sectioned at 90 μm using a vibratome (Leica VT 1000S) and mounted on microscope slides.
III Staining procedure	Sections were rinsed in dH_2O for 2 x 4 minutes and then placed in a mixture containing 10 ml of Solution D**, 10 ml of Solution E**, and 20 ml of dH_2O for 10 minutes. Sections were yet again rinsed in dH_2O for 2 x 4 minutes.
IV Dehydration	Sections were dehydrated in 50%, 75% and 95% ethanol, 4 minutes each and then in absolute ethanol for 4 minutes. Sections were cover slipped.
V Imaging	From each region of interest, 10 layer III pyramidal neurons were selected for imaging and analysis. From each neuron, a segment of 50 μm from a basilar dendrite was imaged in a z-stack (Leica Brightfield microscopy, x100 objective). Colors were inverted and intensity and brightness adjusted.
VI Reconstruction and quantification	Dendrites were reconstructed semi-automatically using image processing program NeuronStudio, with some manual adjustments. Spines were subsequently detected and counted.
VII Statistical analysis	Appropriate statistical measures were applied to the data.

Table 2.3 Description of the Golgi-Cox protocol from the commercially available kit.

* Contain mercuric chloride, potassium dichromate and potassium chromate.

** The protocol does not provide information regarding the content.

Results

The present study aimed to investigate dendritic spine density in Golgi-impregnated layer III pyramidal neurons in the MPFC and the PCC, the main functional hubs of the DMN, in subjects with schizophrenia.

Methodological testing has been a major part of the present study. The Golgi technique has demonstrated varying degrees of success, despite several modifications. Somewhat surprisingly, I was able to achieve reliable staining of all parts of the neurons from brain tissue of the schizophrenia subjects. To the best of my knowledge, no previous studies have successfully performed the Golgi staining technique on tissue of similar age. Conversely, due to methodological issues that will be elaborated on and discussed in later sections, the study failed to obtain viable staining in the control group. A statistical comparison between healthy subjects and subjects with schizophrenia is therefore not available in this pilot study. In the first part of the Results section, images from the schizophrenia subjects will be presented. At the beginning of the study, this group consisted of eight subjects in total. However, four subjects were excluded from the analysis due to insufficient staining. Images from both successful and unsuccessful staining are provided. Furthermore, images from the control group are presented, as well as a description of the methodological problems encountered in the study.

Statistical analyses, namely independent sample T-test and analysis of variance (ANOVA), were applied to the data in order to firstly, compare mean dendritic spine density between the MPFC and the PCC, and secondly, compare the means of every subject in the sample. Because subjects differed in age, each served as an independent age group, and thus age was a measured variable. The results of the statistical analyses may be found further below.

Golgi staining in the schizophrenia specimens

The schizophrenia group initially consisted of brains from eight subjects. The study achieved respectable staining in 4 of these brains, whereas the other four brains were not included in the analysis due to inadequate staining. Images from these samples will be presented first.

Figures 3.1, 3.2., and 3.3 illustrate major methodological problems encountered in the present study. A common byproduct in the Golgi method is that of *overchromation*, in which precipitates of excess silver chromate solution build up. Several microscope slides had to be excluded from imaging and analysis due to dark blobs of precipitates covering the sections, making it difficult to detect neurons (figure 3.1). Precipitation of silver chromate was also present along the surface of the tissue block in some cases (figure 3.2), preventing staining reagents to penetrate the tissue sufficiently. In figure 3.3, a few neurons are stained, albeit the staining is limited to superficial regions of the tissue. When the pH of the chromation solution was adjusted, the problem of shallow staining was reduced, and pyramidal neurons in cortical layer III were stained and visible.

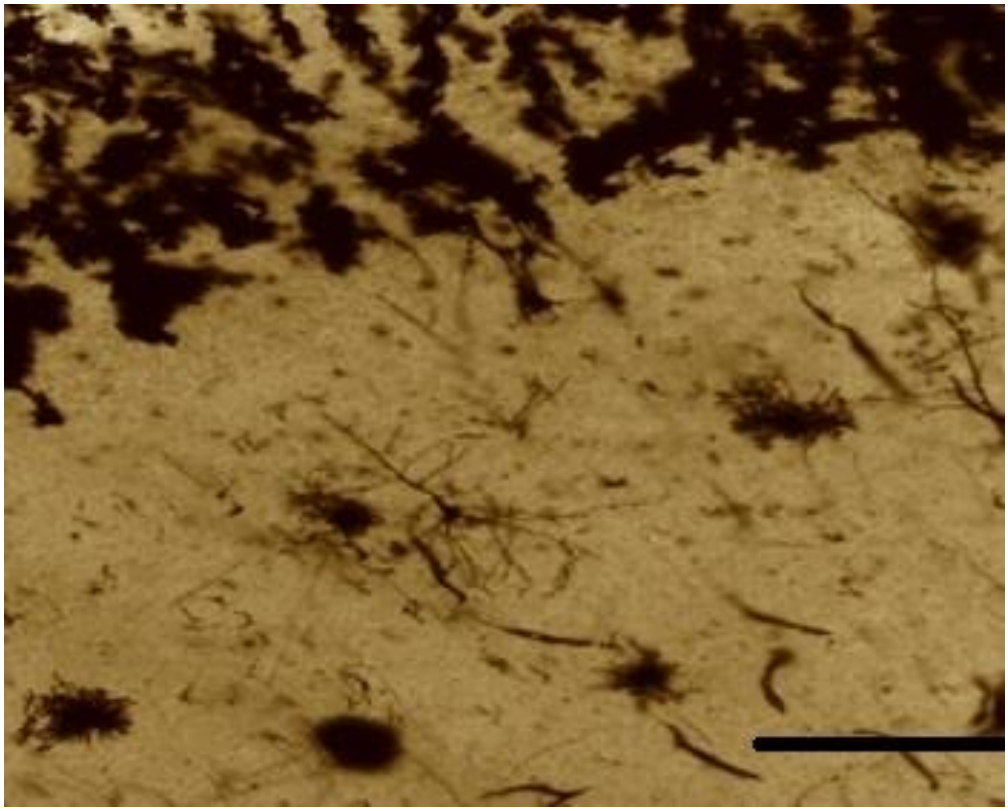

Figure 3.1 Image showing staining artifacts covering the section. Precipitates of silver chromate reagents build up, covering most of the neurons. Scale bar is set to 100 µm.

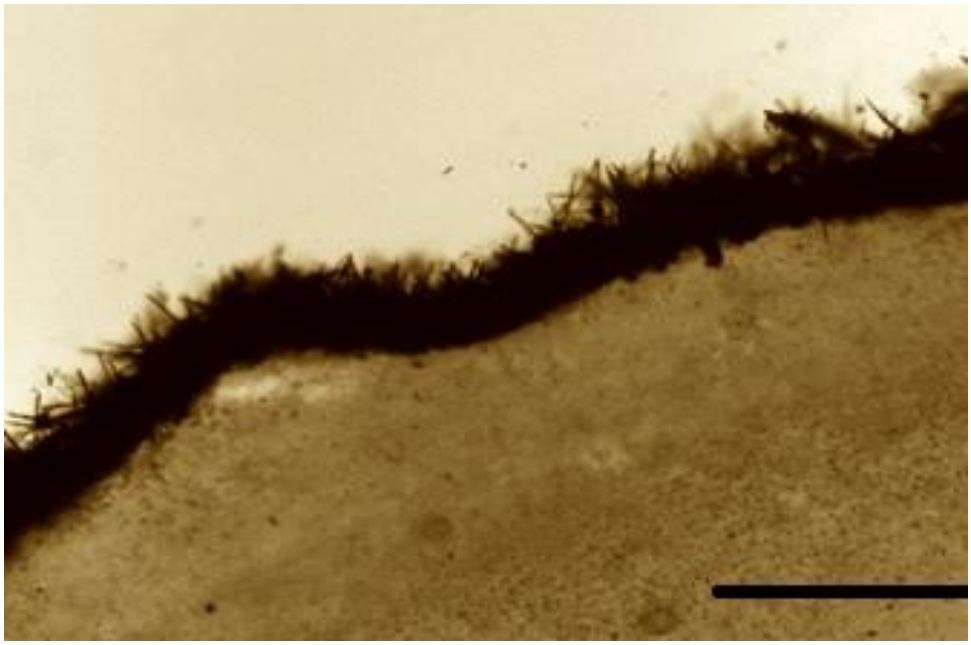

Figure 3.2 Image showing silver chromate precipitations along the surface of the tissue block. Scale bar is set to 100 µm.

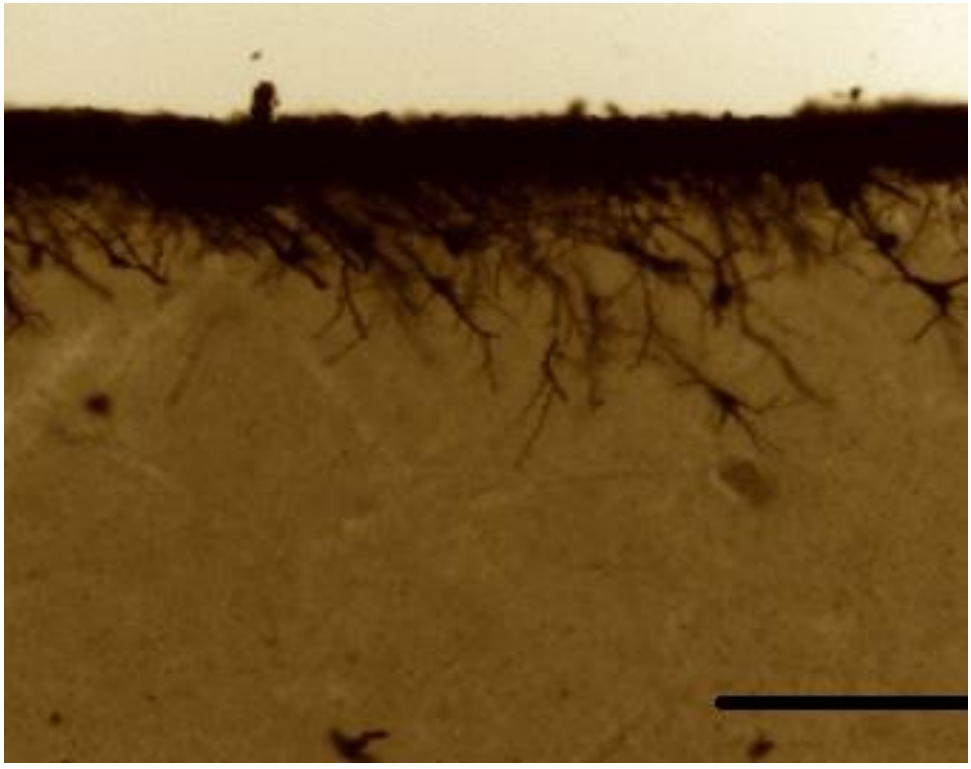

Figure 3.3 Image acquired from the MPFC in the brain of a subject with schizophrenia. Neurons are stained; however, only in the most superficial areas of the tissue. Scale bar is set to 100 µm.

In four of the brains included in the analysis, the Golgi-Kopsch variation developed in the present study impregnated many cells. As evident in figure 4.1, these neurons were completely stained, including soma, dendritic tree and spines. The staining appears to be uniformly distributed across the section, and was successful in reaching deeper areas of the tissue, including layer III (figure 4.2). The sections are not dominated by staining artifacts, and there is high contrast between cells and their background. Cytoplasmic details are not observable.

In the present study, I was especially interested in dendritic spines on pyramidal neurons in layer III, and was able to obtain viable staining (figure 4.3 and 4.4). Spines are clearly visible, including spine neck and head.

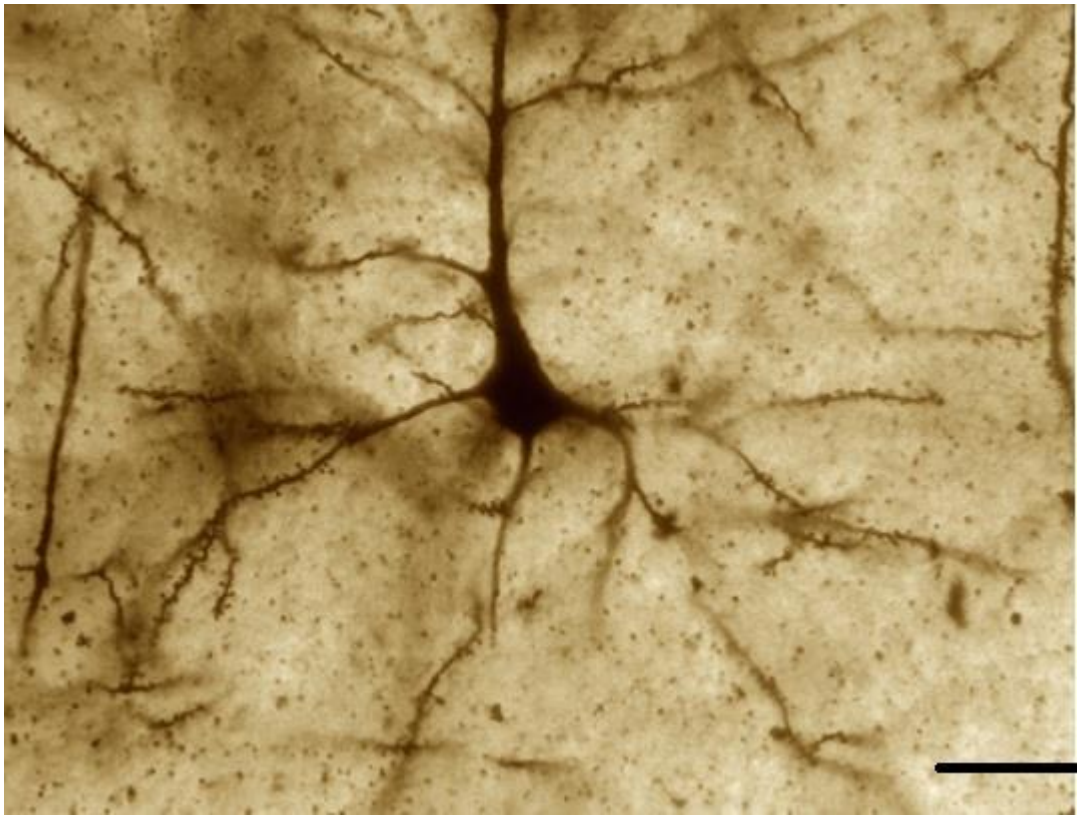

Figure 4.1 Image from a Z-stack showing a Golgi-Kopsch-impregnated layer III pyramidal neuron in the MPFC from a subject with schizophrenia. The neuron is completely stained. Scale bar is set to 20 µm.

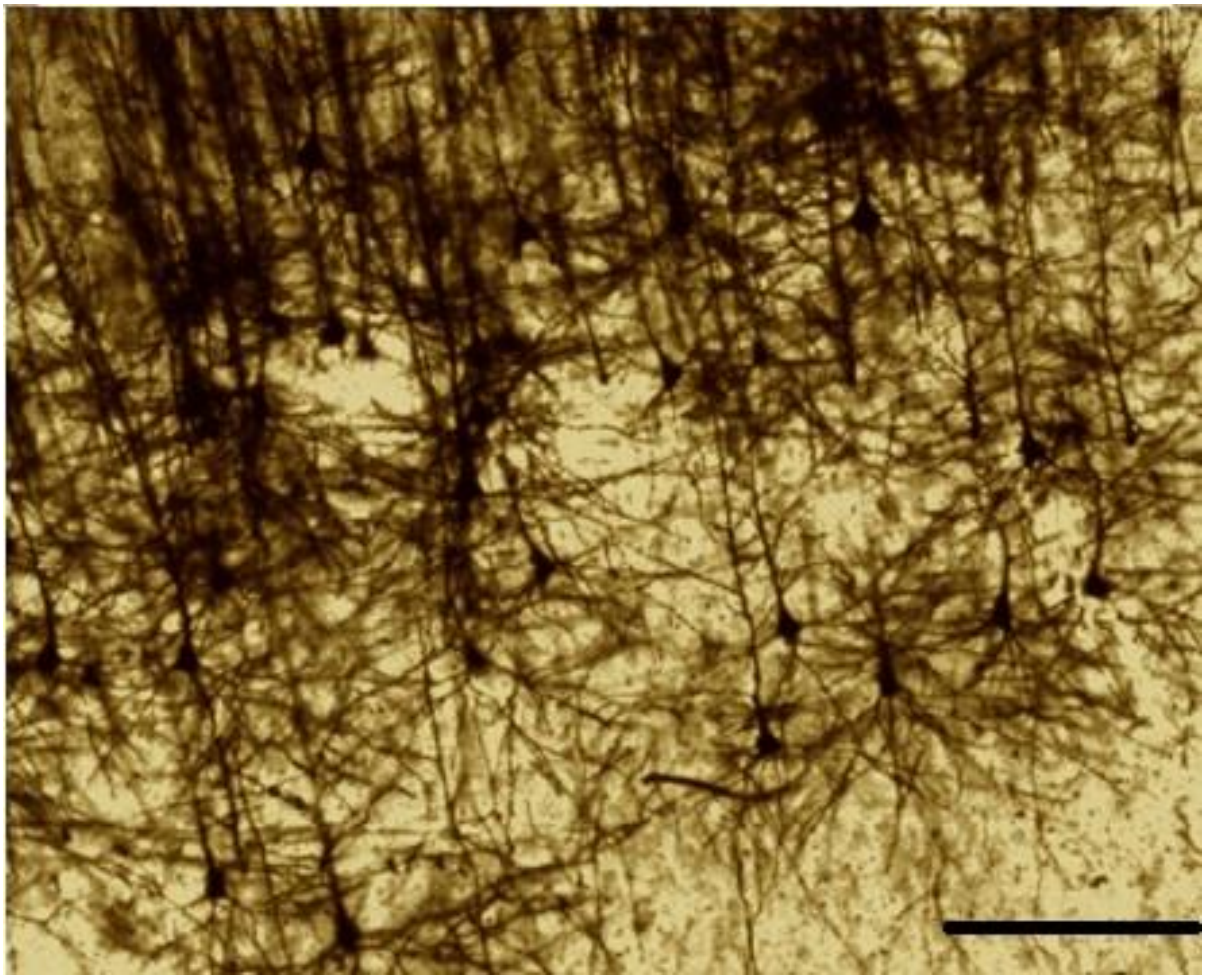

Figure 4.2 Image from layer III of the MPFC from a subject with schizophrenia. Neurons are uniformly distributed across the section. Scale bar is set to 100 µm.

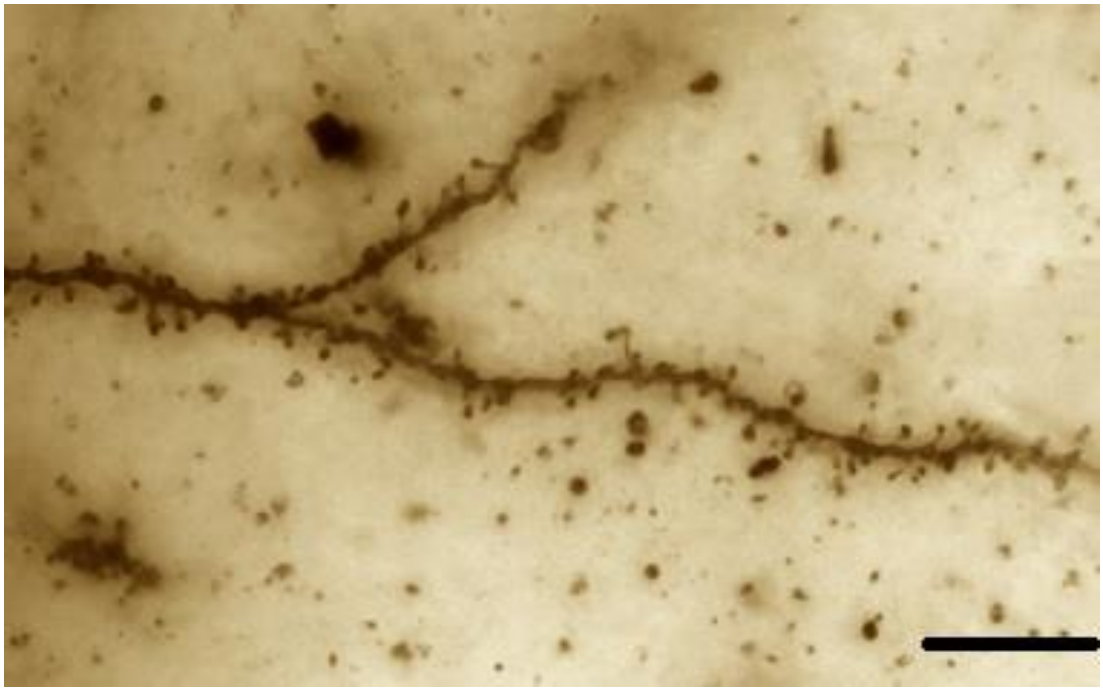

Figure 4.3 Image from a subject with schizophrenia, showing a branching dendrite in the PCC. Spines are clearly visible. Scale bar is set to 10 µm.

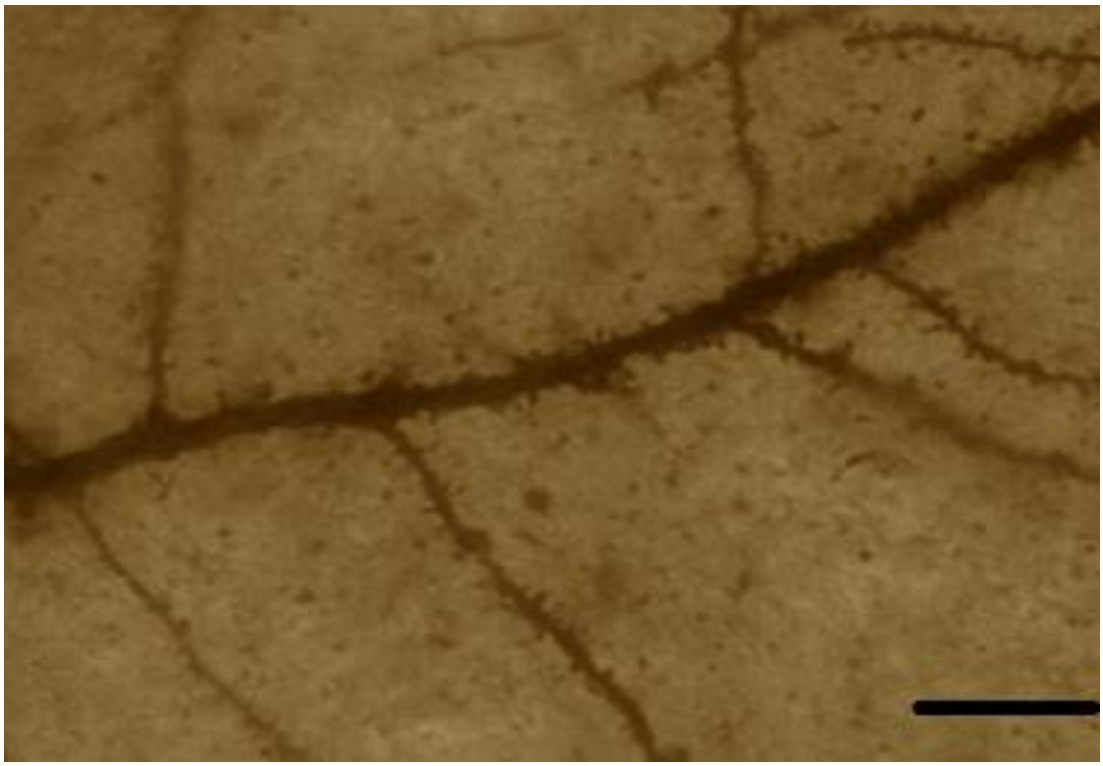

Figure 4.4 Branching dendrite with spines in the MPFC from a subject with schizophrenia. Scale bar is set to 10 µm.

Golgi staining of the control brain tissue

The present study failed to obtain any viable staining with the Golgi technique in the healthy control group. In the schizophrenia group, unsuccessful staining was mainly related to staining artifacts and was improved by adjustments to the protocol. The difficulties encountered when staining the control material were of a different nature. While the Golgi-Cox and the Golgi-Kopsch variation failed to show any visible staining at all, the rapid Golgi technique did indeed lead to some viable staining of neurons. However, only soma and apical dendrites were impregnated (figure 5.1). Visible basilar dendrites were infrequent and mostly lacking and, importantly, few spines could be detected (figure 5.2). Consequently, I was not able to obtain any data from the healthy control group in this pilot study. The reasons for this will be discussed later in the thesis.

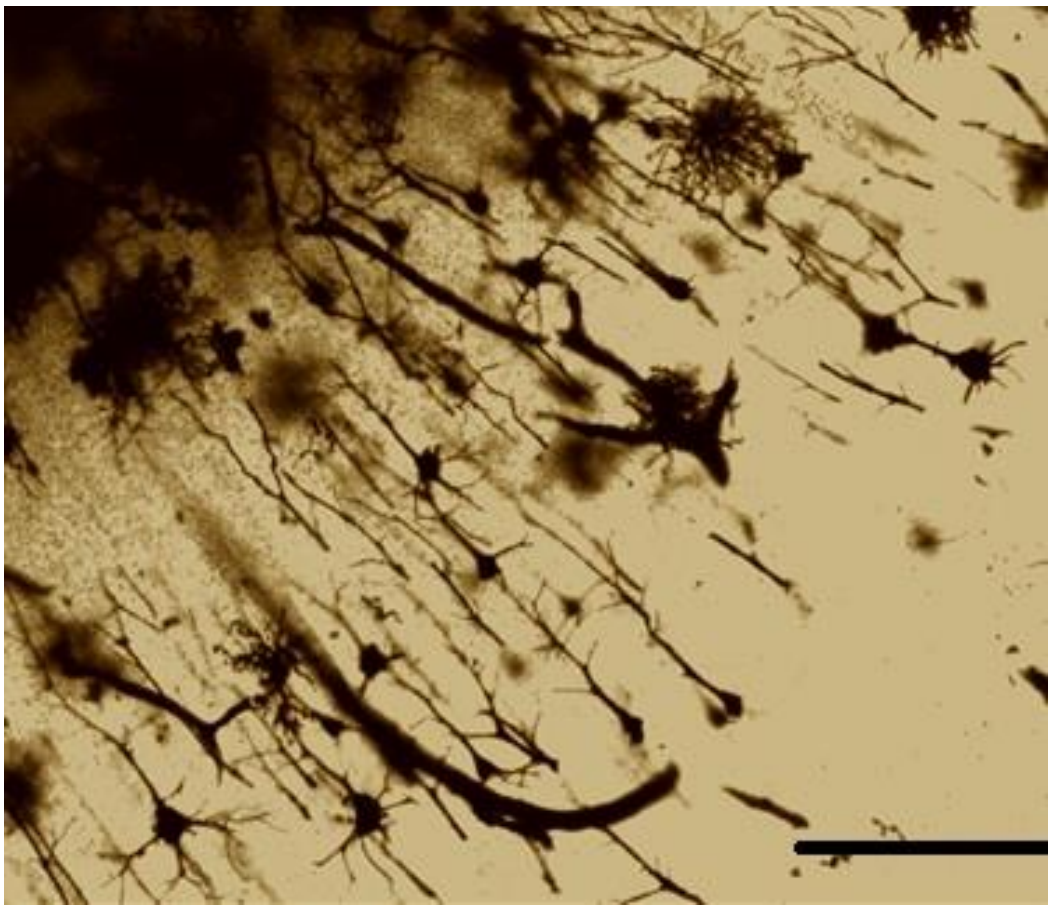

Figure 5.1 Image acquired from the MPFC of a control subject, showing neurons impregnated with the rapid Golgi technique. While soma and apical dendrites are visible, no basilar dendrites could be detected. Scale bar is set to 100 µm.

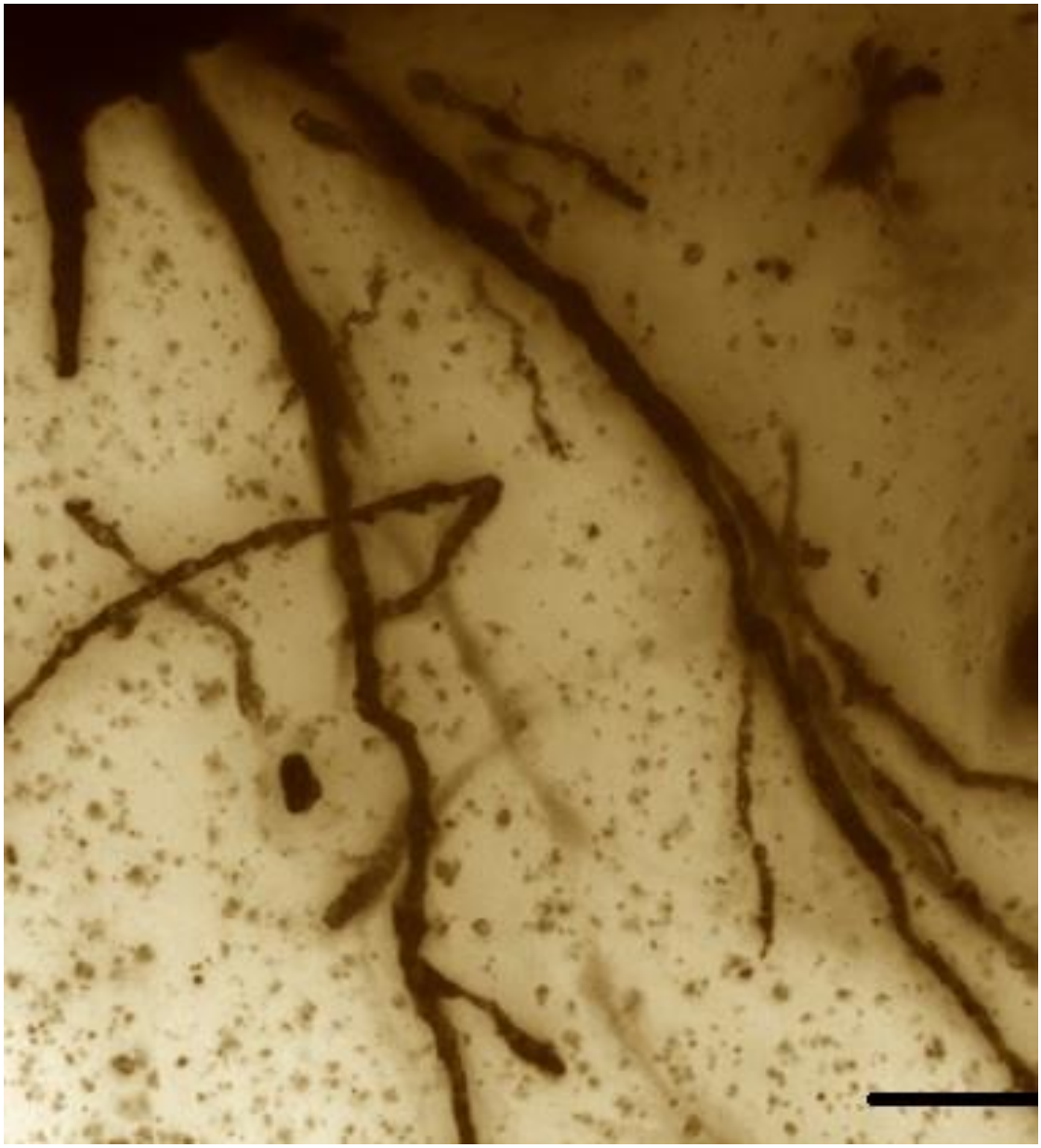

Figure 5.2 Image from the MPFC from a control subject showing dendrites impregnated with the rapid Golgi technique. Few spines are noticeable. The soma is visible in the upper left corner. Scale bar is set to 10 µm.

Dendritic spine density in the functional hubs of the DMN

Mean dendritic spine densities in the two functional hubs of the DMN were compared using the independent samples T-test. A summary of mean spine density, as well as a statistical description, is provided in table 3.1 and figure 6.1. The present study detected a significant difference in mean dendritic spine density at the $p < .01$ level between regions ($p=0.003$), with the MPFC showing the highest spine density (0.87±0.20/µm), while the PCC had a mean spine density of 0.74±0.17/µm. The presence of outliers in the data was checked by inspecting the histogram and box plot. The data for the MPFC and the PCC included one score each that was identified as an outlier; however, both were considered to lie within the range of plausible scores. By inspecting the difference between the mean value and the 5% trimmed mean, the potential threat of outliers was assessed. The mean value and the 5% trimmed mean did not differ significantly in either analyses, and the outliers were therefore not regarded as a threat, and were not excluded from the analysis.

Dendritic spine densities in the MPFC and the PCC were also compared within the same brain of each subject using the independent samples T-test. A summary of mean spine density, as well as statistical description is provided in table 3.2. In case ID 3647, 3739, and 2837, mean spine density differed significantly at the $p < .05$ level, with the MPFC having the highest spine density in all three subjects (1.07±0.16/µm, 0.81±0.12/µm and 0.87±0.15/µm, respectively). As opposed to the other subjects, the PCC in case ID 1827 showed a slightly higher numerical mean spine density than the MPFC (0.79±0.11/µm and 0.73±0.18/µm, respectively), but this difference did not reach statistical significance.

REGION	*MEAN SPINE DENSITY*	*SD*	*SE*	*95% CI*
Posterior cingulate cortex	0.74	0.17	0.03	0.69 – 0.80
Medial prefrontal cortex	0.87	0.20	0.03	0.80 – 0.93

Table 3.1 Mean dendritic spine density in the MPFC and the PCC. Descriptive statistics are given, including the standard deviation, standard error, and 95% confidence interval. A significant difference between means was detected (p = 0.003).

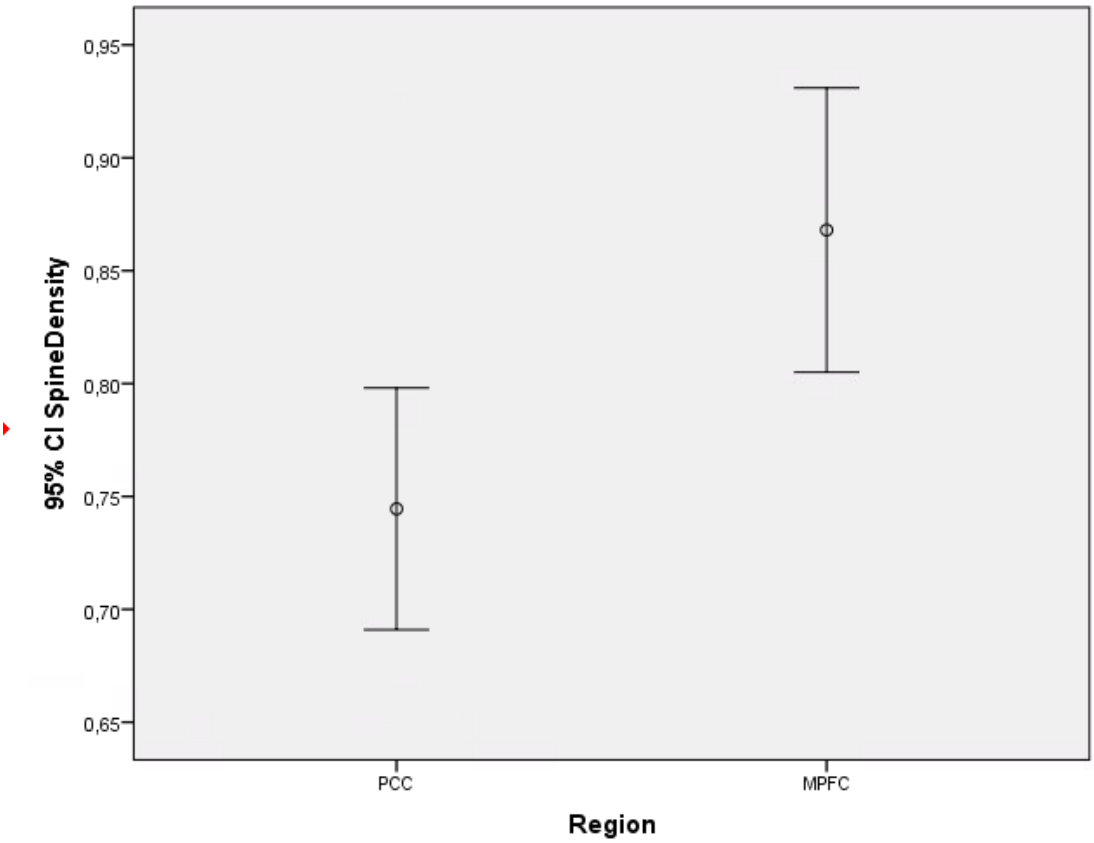

Figure 6.1 Error bar plot displaying mean dendritic spine density in the MPFC and the PCC. Error bars show the 95% confidence interval.

CASE ID	*REGION*	*MEAN SPINE DENSITY*	*SD*	*SE*	*0.95% CI*	*SIG.*
3647	MPFC	1.07	0.16	0.05	0.95 – 1,19	0.030
	PCC	0.91	0.17	0.05	0.80 – 1.01	
3739	MPFC	0.81	0.12	0.04	0.72 – 0.89	0.001
	PCC	0.61	0.10	0.03	0.54 – 0.68	
2837	MPFC	0.87	0.15	0.05	0.76 – 0.97	0.005
	PCC	0.67	0.13	0.04	0.57 – 0.76	
1827	MPFC	0.73	0.18	0.06	0.60 – 0.85	0.322
	PCC	0.79	0.11	0.04	0.71 – 0.87	

Table 3.2 Mean dendritic spine density in the MPFC and the PCC within each subject. Numbers are given as spines per µm. Descriptive statistics are provided, including standard deviation, standard error, 95% confidence interval, and p-value.

Dendritic spine density in different age groups

A one-way between-groups ANOVA was conducted in order to explore a possible effect of age on dendritic spine density. Each participant served as an individual age group, (case ID 1827: 33 years, case ID 2837: 46 years, case ID 3647: 55 years, case ID 3739: 67 years). There was a statistically significant difference at the $p < .01$ level for the four age groups: $F(3, 76) = 12.2$, $p = 0.001$. Post-hoc comparisons using the Bonferroni test indicated that the mean spine density for case ID 3647 (0.99±0.17) was significantly different from the three other test subjects. Case ID 3739 had the lowest mean spine density (0.70±0.15); however, this did not differ significantly from the mean spine densities for case ID 1827 and 2837. A summary of mean dendritic spine density and statistical description is provided in table 4.1 and figure 7.1.

CASE ID	*AGE*	*MEAN SPINE DENSITY*	SD	SE	95% CI
3647	***55***	0.99	0.17	0.04	0.91 – 1.07
3739	***67***	0.70	0.15	0.03	0.64 – 0.78
2837	***46***	0.77	0.17	0.04	0.69 – 0.85
1827	***33***	0.76	0.15	0.03	0.69 – 0.83

Table 4.1 Mean spine density in the DMN in four subjects with schizophrenia. Each subject served as one age group. Statistical description, including standard deviation, standard error and 95% confidence interval, is provided.

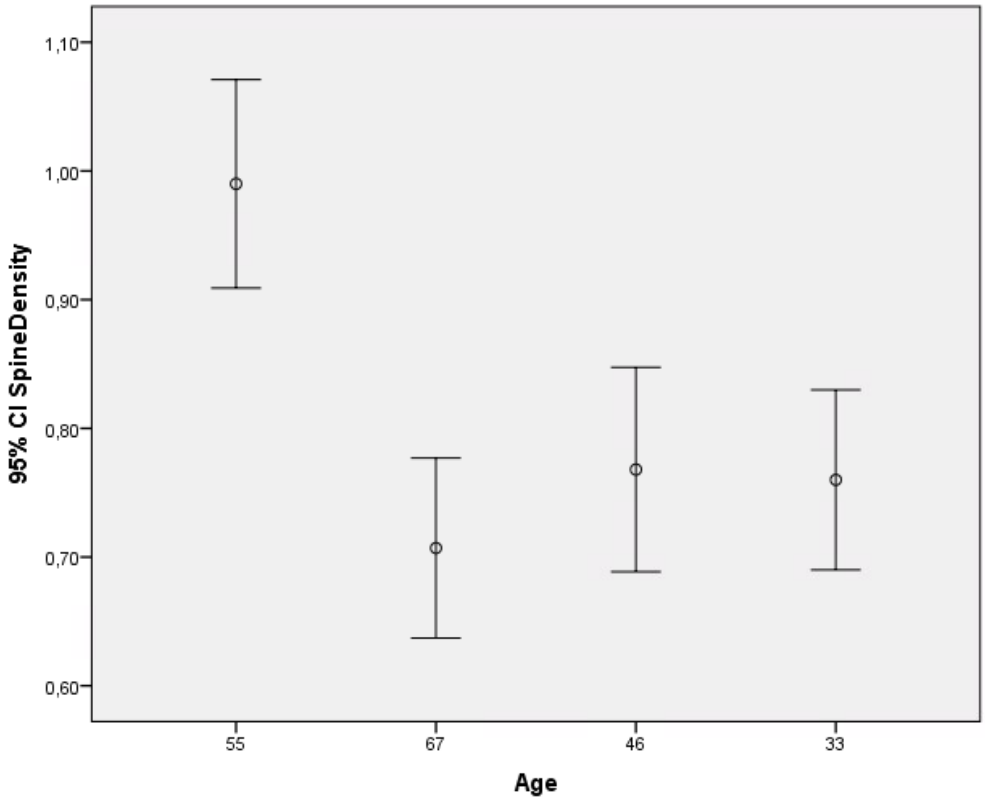

Figure 7.1 Error bar plot showing the mean dendritic spine density in the DMN in four subjects with schizophrenia. Each subject serves as one age group. Error bars show the 95% confidence interval.

Discussion

Main findings

In the present study, dendritic spine density on layer III pyramidal neurons in the MPFC and the PCC in medication-naïve subjects with schizophrenia was investigated. These areas are regarded as the main functional regions in the DMN. The DMN is responsible for spontaneous, episodic, self-reflective cognition, and a growing body of knowledge suggests involvement of this network in the pathology of schizophrenia. Previous fMRI studies have demonstrated abnormally high functional activity and connectivity of the DMN in patients with schizophrenia. The present study aimed to investigate the synaptic correlate for the increased functional connectivity reported by several neuroimaging studies. With the general supposition that structure is the basis for function, I hypothesized that dendritic spine density in the DMN would be increased in schizophrenia. Moreover, the study also aimed to investigate potential differences in spine density between the MPFC and the PCC. The results of the study demonstrated a significant difference, with higher spine density in the MPFC in total. Individual analyses also demonstrated significantly higher mean spine density in the MPFC compared to the PCC in three of the subjects included in the study. For the last subject, the PCC had a higher numerical spine density compared to the MPFC; however, the means did not differ significantly.

The study also assessed a potential effect of age on spine density. I hypothesized that mean spine density would be negatively correlated with age, as suggested by some studies. The oldest subject in the sample (age 67) had the lowest numerical mean spine density; however, this mean did not differ significantly from that of the subjects aged 33 and 46. Additionally, the mean spine density for the subject aged 55 was significantly higher than that of the other age groups, disagreeing with the hypothesis. A major limitation in the present study was the lack of staining of the control brain tissue. For this reason, this thesis was not able to include a statistical comparison between healthy and schizophrenic subjects. Nevertheless, the results of the study will be discussed in light of similar studies from healthy subjects as well as subjects with schizophrenia, and lastly, findings from animal studies.

Interpretation of the results

Regional differences in dendritic spine density

The present study found significantly higher dendritic spine densities in the MPFC compared to the PCC, even within the same brain. Regional differences in the present study is in line with existing literature suggesting more complex and extensive connectivity within the frontal lobes compared to other cortical areas (Uylings et al., 1990). Elston (2000) compared spine density on pyramidal cells in layer III of frontal, temporal, occipital and parietal lobes in primates, and found that prefrontal pyramidal cells were significantly more spinous than cells in other areas. Compared to the primary visual area (BA 17), prefrontal cells had 16 times more spines while the differences were less pronounced in other regions. Jacobs et al. (2001) compared dendritic morphology between several different Brodmann areas (1-4, 6, 10-12, 22, 39 and 44) in humans, and found that BA10 (MPFC) was significantly more complex in terms of e.g., branching, and most notably, spines (figure 8.1).

The larger number of spines in the MPFC is in line with evidence suggesting that this region has a large capability, and a large demand, for integrating excitatory input (Uylings et al., 1990). As one of the main regions of the DMN, area 10 is part of the so-called *social brain*, responsible for social cognition (Mars, 2012). Smallwood et al. (2012) have proposed that since our social environments are continuously changing, *the social brain* must perhaps have higher abilities and demands for adaptation and plasticity. High dendritic spine density is consistent with this notion, as morphological changes to dendritic spines are associated with synaptic plasticity (Yuste and Bonhoffer, 2001).

In one of the subjects included in the study, the numerical mean spine density was higher in the PCC than in the MPFC, albeit, without statistical significance. A plausible explanation for this difference could perhaps be ascribed to the impact of psychosocial factors. Dendritic spines are plastic structures that have been shown to change in response to various conditions in experimental animals (Bryan and Riesen, 1982). Schizophrenia show high co-morbidity with other mental illnesses, such as anxiety and depression (Buckley et al., 2009), and subjects with schizophrenia have increased risk of suicide (Hawton et al., 2005). It is therefore reasonable to assume that schizophrenia is associated with high levels of stress. Stress-induced alterations in spine morphology in the MPFC have been reported (Radley et al., 2008, Moench and Wellman, 2015). Radley et al. (2006) found that repeated stress resulted in a decrease of layer II/III dendritic spines in the MPFC in rats. Furthermore, the schizophrenia patients in the sample were hospitalized for long periods at a time. Studies

have indicated a positive correlation between environmental enrichment and spine growth (Leggio et al., 2005, Jung and Herms, 2012). The effects of social deprivation in humans are unknown; however, in rats, social isolation has a negative effect on spine density (Silva-Gomez et al., 2003). However, as subjects in the sample all have the same diagnosis, and were all hospitalized, it can be argued that the effects of these variables would be equal for all subjects and would therefore not contribute to individual differences in spine density.

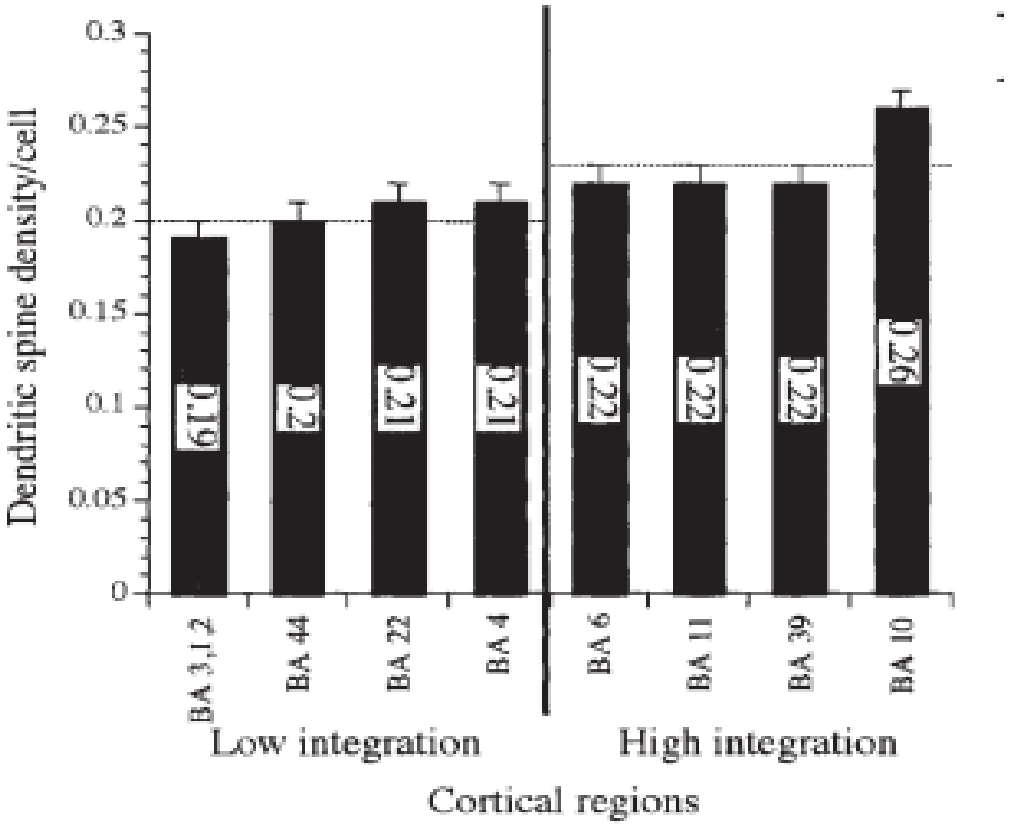

Figure 8.1 Bar graph adopted from Jacobs et al. (2001) showing mean dendritic spine density in different Brodmann areas. Numbers are given as spine density per µm. BA 10 show the highest spine density (0.26/µm). Additionally, other cortical regions associated with high integration display higher spine density than low integration areas.

Studies investigating dendritic spine density in regions beyond the MPFC and the PCC in schizophrenia may be found in table 5.1. Garey et al. (1998) and Glantz and Lewis (2000) have explored spine density in layer III of the DLPFC. Both studies report significant differences in mean spine density between healthy controls and schizophrenic subjects, and the means for the DLPFC are significantly lower than the numbers obtained in the present study. While this finding could possibly support the hypothesis that, in schizophrenia, synaptic connectivity is increased in the regions corresponding to the DMN, it could also be interpreted as evidence supporting the notion of regional differences in spine density in the brain in general. Nevertheless, it is intriguing that even within the PFC, adjacent areas show substantial differences in spine density in schizophrenia. This might imply that the pathology in schizophrenia is more heterogeneous across regions than previously believed.

Another plausible factor accounting for the lower mean spine density obtained in the studies by Garey et al. (1998) and Glantz and Lewis (2000), is neuroleptic medication. One of the major concerns when conducting research on brain material obtained from schizophrenic subjects is the possible confounding effect of neuroleptic drugs. In rats, the administration of antipsychotic medication, Haloperidol, has been shown to contribute to structural changes in synapses (Benes et al., 1985). In the studies by Garey et al. (1998) and Glantz and Lewis (2000), the schizophrenia sample consisted of medicated subjects and thus the contribution of neuroleptics cannot be ruled out or evaluated. Glantz and Lewis (2000) controlled for the effect of medicine by comparing the means of healthy and schizophrenic subjects to that of non-schizophrenic psychiatric patients who were on antipsychotic medication. The mean spine density did not differ significantly between these patients and the healthy control group. However, as the total exposure to neuroleptic drugs is likely to be more extensive for the schizophrenic subjects than for psychiatric patients experiencing single episodes of psychosis, it is reasonable to believe that neuroleptic drugs will have a greater impact on the schizophrenic subjects as a function of time. Therefore, the effect of antipsychotic medication in these studies cannot be excluded from the analysis. The present study, however, was conducted on medication-naïve schizophrenic subjects as neuroleptic drugs were yet to be discovered in the thirties and forties. One possibility is that the difference between the observed mean spine density in the MPFC and the DLPFC could be ascribed to antipsychotic medication.

Although the present study demonstrated significantly higher mean spine density in the MPFC (0.87/µm), the corresponding number for the PCC (0.74/µm) is also a relatively

high number, when compared to numbers provided by Jacobs et al. (2001) from other cortical regions (figure 8.1). This is consistent with existing literature on the DMN, suggesting high functional connectivity in and between the associated regions (Khalsa et al., 2013). Additionally, high spine density is also consistent with studies that have observed high white matter connectivity in DMN using DTI (Greicius et al., 2009). It is possible, then, that the results of the present study are supportive of high structural connectivity in the DMN *in general*, and not in schizophrenia specifically. Therefore, a direct comparison of dendritic spine density in the DMN between subjects with schizophrenia and healthy subjects is necessary to further elucidate the implication of this finding.

Differences in dendritic spine density between healthy and schizophrenic subjects

The ultimate goal of this thesis was to compare dendritic spine densities in the MPFC and the PCC between healthy control subjects and subjects with schizophrenia, driven by the hypothesis that spine density would be increased in schizophrenia. However, the present study was not able to obtain any viable results of the Golgi staining in the control group, and a statistical comparison is thus not available. Nevertheless, findings from existing literature on spine densities in healthy brains and schizophrenic subjects may shed some light on the results of this study.

Jacobs et al. (1997) investigated age-related differences in dendritic spine density in layer III pyramidal neurons in BA 10 (MPFC) in post mortem human brain material obtained from healthy subjects. The mean spine density for subjects under the age of 50 was 0.24/µm, while the corresponding number for subjects aged 50 and up was 0.13/µm. In the aforementioned study by the same authors (Jacobs et al., 2001), mean spine density in BA 10 was found to be 0.26/µm. These numbers are significantly lower than the means obtained in the present study. For the schizophrenia sample, the mean spine density was 0.87/µm in area 10. Although caution should be made when comparing numbers from different studies, the higher mean spine density in BA 10 obtained in the present study might support the hypothesis that synaptic connectivity is increased in regions that also show abnormally high functional connectivity in schizophrenia. On a general note, this finding may be an important contribution to the growing body of knowledge suggesting dendritic spine pathology as a key characteristic of several psychiatric diseases (Penzes et al., 2011). The results of the study are in line with studies that have explored altered spine distribution in hyperfunctional networks

in ASD. ASD has been associated with an increase in spine densities in regions that also show hyperfunctional connectivity in fMRI studies (Hutsler and Zhang, 2010).

Moreover, the finding of the present study provides support to the hypothesis proposed by Gaspar et al. (2009) suggesting that abnormal brain connectivity in schizophrenia may adhere to different patterns in different regions. While some regions display reduced synaptic and functional connectivity, other regions may show the opposite arrangement. Lastly, there is a general lack of studies examining structure-function relations using gray matter, especially in regards to the DMN. The finding of the present study suggests that fluctuations in functional connectivity may not just reflect white matter structure, but also gray matter structural connectivity. Exploring the correspondence between functional connectivity and gray matter measures may contribute greatly to the understanding of the human connectome.

To the best of my knowledge, studies investigating dendritic spine density in the PCC in humans are lacking. Elton et al. (2005) explored spine density in the PCC in macaque monkeys. Studies have reported that spine distribution differs according to species (Benavides-Piccione et al., 2002), and so direct inference is not sustainable. Albeit, it is quite interesting that Elston et al. (2005) observed a mean spine density of 1.39/µm on layer III pyramidal neurons of the PCC of macaque monkeys. Additionally, a previous study from our laboratory examined spine density in the PCC in rats and found the spine density to be 1.64/µm (Pesqueira, 2015), although this was in layer V, and not layer III. These findings indicate that the spine density in the PCC in the schizophrenia sample in this study (0.74±0.17/µm) is abnormally low. This could perhaps be interpreted as further evidence that pathology in schizophrenia affects brain regions differently. While the MPFC has a profound increase in synaptic connectivity, the PCC displays the opposite pattern, perhaps as compensation to regain homeostasis in the network. Nevertheless, to further elucidate the functional implication of this finding, a statistical comparison between subjects with schizophrenia and healthy controls is highly needed.

The effect of age on spine density

Lastly, the present study aimed to investigate a possible effect of age on spine density. Alterations in neuronal morphology, including synaptic reorganization, have been found to occur in normal aging, and is related to cognitive and memory decline (Dickstein et al., 2012). The results of this study did not show a significant difference in spine density between

age groups, but then again the sample size (n=4) was too small to reveal a significant effect. Previous studies have implicated that the DMN is especially vulnerable to the effects of aging (Damoiseaux et al., 2008). This was evident in the aforementioned study by Jacobs et al. (1997), in which spine density in subjects below age 50 differed significantly from the spine density of subjects aged above 50.

STUDY	*SPECIES*	*REGION*	*MEAN SPINE DENSITY*
JACOBS ET AL. (1997)	Human,	BA 10	0.24/µm
GAREY ET AL. (1998)	Human, schizophrenic subjects, healthy controls	BA 10, 11, 45	Numbers are given as a total for all three regions: Schizophrenia subjects: 0.1/µm Normal controls: 0.3/µm
JACOBS ET AL. (2001)	Human	BA 10	0.26/µm
GLANTZ AND LEWIS (2000)	Human, schizophrenic subjects, healthy controls	BA 46	Schizophrenia subjects : 0.26/µm Normal controls: 0.33/µm
ELSTON ET AL. (2005)	Macaque monkeys	BA 23	1.39/µm
PESQUEIRA (2015)	Rats	BA 23	1.64/µm

Table 5.1 Dendritic spine densities in frontal regions central to the present study in schizophrenic subjects and healthy controls. Due to the lack of similar studies on the PCC in humans, studies from primates and rats are also included.

The relation between function and structure

The present study aimed to explore the structural correlate for increased functional connectivity in the DMN as observed in neuroimaging studies of schizophrenia. I hypothesized that increased functional connectivity would be associated with increased synaptic connectivity. Hence, a presupposition of this thesis is a strong relation between function and structure. There is strong evidence for a structure-function association in the brain (Sui et al., 2014). However, in some cases, brain regions do not have to have direct structural connections in order to display functional connectivity (Biswal et al., 2010) and furthermore, a direct structural link between different brain areas does not necessarily lead to a functional relation. Hence, function may not necessarily be reflected in structure. Moreover, inferring function from structure and vice versa might not be attainable in every case. Nonetheless, several studies have indeed demonstrated a significant correlation between structural and functional connectivity in the DMN (Greicius et al., 2009, Khalsa et al., 2013, Horn et al., 2014), making it plausible that abnormally high functional connectivity in schizophrenia could be associated with increased spine density on dendrites receiving input from other DMN regions.

Methodological limitations

Post mortem fixation delay

A major limitation of the present study is the lack of control brain material for the statistical analyses, which weakens the study's empirical impact. A total of eight brains from healthy control subjects were at our disposal, provided by the University of Oslo. However, due to methodological issues related to the Golgi staining technique, no viable results were obtained. I believe that the lack of stained spines is primarily due to long post mortem interval (PMI). PMI before preservation has been shown to be a crucial determinant of successful staining, and a prolonged interval may heavily affect the quality of the brain tissue (Williams et al., 1978, DeRuiter, 1983). For the control material in this study, PMI was approximately 1 week, and this most likely had a deteriorating effect on the tissue. Williams et al. (1978) argue that when post-fixation is delayed with 6 hours or more, degenerative changes supervene and may alter the pattern of impregnation. Although the Golgi technique managed to stain neuronal cell bodies to some extent, no spines were observed. It is possible that while soma, and to a certain degree dendrites, remain intact, spines are more sensitive to

post mortem fixation delay. Preserved information regarding autopsy and fixation of the schizophrenia sample in the present study is scarce; however, in some of the medical records PMI has been stated. For three of the subjects, PMI was as little as a few hours, which further supports the hypothesis that post mortem fixation delay has detrimental effects for the Golgi staining technique.

Reconstruction of dendrites

The reconstruction of dendrites and the subsequent quantification of spines were conducted using NeuronStudio. This program enables the construction of a maximum intensity projection that resembles the 3D structure of the dendrite. This process is semi-automatic and relies, to some degree, on the researcher performing the analysis. It may therefore be argued that the quantification of spines is susceptible to biases. Ideally, the spine counting should be "blind"; that is, it should be executed by an independent researcher not involved in the project.

Issues concerning the use of post mortem tissue

Post mortem studies of brains from subjects with schizophrenia have allowed researchers to explore the structural correlates of abnormal functional processes, and have revealed several key characteristics central to the pathology in schizophrenia. There are, however, a few matters when conducting post mortem studies that should be addressed and carefully considered. Caution should be made when trying to infer causality from histological findings in postmortem tissue. The neuronal alterations in the context of schizophrenia could represent either cause, consequence, compensation or confound. Post mortem studies *per se* offer no insight into what category histological findings fall into. There are, however, some indications that spine pathology is primary to the disorder. Cortical thinning and dilation of the ventricles may be observed at the time of first diagnosis (Steen et al., 2006), suggesting that synaptic reorganization is a cause rather than a neurotoxic effect from illness or medication.

Further research

In the present study, the finding of high dendritic spine density in the MPFC stands out as particularly relevant for further research. Compared to numbers obtained from healthy

subjects in previous studies, the MPFC showed increased dendritic spine density in the schizophrenia sample in this study. A direct comparison of spine density in the DMN between healthy controls and subjects with schizophrenia is highly needed in order to further elucidate the implication of the results obtained in the present study. Moreover, immunocytochemical techniques could corroborate the findings of the study. If the hypothesis that spine density is increased in the DMN proves correct, it would be interesting to investigate the expression of key presynaptic and postsynaptic proteins in the MPFC and the PCC. Additionally, the present study aimed to investigate the potential effect of age on spine density in the DMN; however, the results were inconclusive. In order to determine age-related changes, a prospective study will require a substantially larger sample size.

Concluding remarks

In this thesis, I have investigated synaptic connectivity in the DMN in schizophrenia. The results indicate regional differences in dendritic spine density within the network, with the MPFC showing significantly higher spine density than the PCC. Additionally, when compared to findings from healthy subjects in other studies, the MPFC demonstrated significantly higher spine density in the schizophrenia sample included in the present study. This result would suggest that synaptic connectivity in the DMN is increased in schizophrenia, by means of increased dendritic spine density. This is in accord with fMRI studies demonstrating increased functional connectivity within this network. Thus, the prevailing view that schizophrenia merely involves decreased structural connectivity may need revision. The PCC showed significantly higher spine density than other cortical areas, however no direct comparison between schizophrenia patients and healthy subjects was available, and these results remain inconclusive. Lastly, the present study found no effect of age on spine density, although the small sample size in the study might have prevented such an effect to be observed. While further studies are required to elucidate the implication of the main results, the present study may be an important contribution to the large body of knowledge suggesting a key role of dendritic spine pathology in the etiology of schizophrenia.

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Appendix 1 Letter of approval from the Regional Committees for Medical and Health Research Ethics (REK)

REK
REGIONALE KOMITEER FOR MEDISINSK OG HELSEFAGLIG FORSKNINGSETIKK

Region:	Saksbehandler:	Telefon:	Vår dato:	Vår referanse:
REK sør-øst	Tor Even Svanes	22845521	28.06.2013	2012/1948/REK sør-øst C
			Deres dato:	Deres referanse:
			14.05.2013	

Vår referanse må oppgis ved alle henvendelser

Ole A. Andreassen

2012/1948 Psykoselidelser uten medikamenter. Bruk av Dr. Gjessings samling.

Prosjektleder: Ole A. Andreassen
Forskningsansvarlig: Oslo universitetssykehus

Vi viser til søknad om forhåndsgodkjenning av ovennevnte forskningsprosjekt. Søknaden ble behandlet av Regional komité for medisinsk og helsefaglig forskningsetikk (REK sør-øst) i møtet 13.06.2013. Vurderingen er gjort med hjemmel i helseforskningsloven (hfl.) § 10, jf. forskningsetikklovens § 4.

Prosjektomtale
Dr. Gjessing var en banebrytende forsker på alvorlige psykiske lidelser. Gjennom sin forskning samlet han i perioden 1923-1948 et stort materiale med urinprøver fra pasienter som da var innlagt på Dikemark sykehus, flere hundre godt bevarte urinflasker. I tillegg ble det samlet inn 32 hjerner fra pasienter som ble lagret med konservering. Dette planla han skulle brukes i fremtidig forskning når nye metoder var blitt tilgjengelige. Vi ønsker nå å gjøre moderne biokjemiske analyser av urinprøvene for å undersøke sammenheng mellom sykdomsutvikling (fra journalopplysninger) og markører i urin, samt bruke moderne histologiske og histokjemiske analyser av hjernene, for å undersøke hjerneorganiske sammenhenger med alvorlig psykiske lidelser. Prosjektet vil gi informasjon om mulige mekanismer ved alvorlige psykiske lidelser, uten påvirkning fra psykosemedisiner, som ikke var i bruk på tiden materialet ble samlet inn.

Saksgang
Komiteen behandlet prosjektet første gang i møtet 29.11.2012, og utsatte den gang å fatte vedtak. Komiteen uttalte at *prosjektet skal undersøke et unikt materiale, og har potensiale til å være nyttig. Samtidig reiser prosjektet en rekke forskningsetiske problemstillinger, ettersom det skal gjøre bruk av journalopplysninger, urinprøver og hjerner fra obduksjon fra pasienter som ikke har avgitt samtykke til at det forskes på dem.*

Komiteen besluttet å innhente en uavhengig uttalelse knyttet til prosjektets særegenhet: *På grunn av materialets historiske natur, reiser prosjektet ikke bare tradisjonelle medisinsk-forskningsetiske problemstillinger, men også problemstillinger knyttet til bruk av sjeldent og følsomt historisk materiale mer generelt. Etter avtale med Nasjonalt utvalg for vurdering av forskning på menneskelige levninger (Skjelettutvalget), oversendes derfor saken til dem for en vurdering før komiteen treffer sin beslutning.*

Samtidig ble prosjektgruppen bedt om å gi sine vurderinger av de merknadene komiteen kommuniserte i vedtaksbrevet av 19.12.2012, med spesiell oppmerksomhet rettet mot destruksjon av materialet, lignende erfaringer fra andre land og hjemmelsgrunnlaget for obduksjon og oppbevaring av hjernene.

Komiteen mottok uttalelsen fra Skjelettutvalget 04.01.2013.

Besøksadresse: Gullhaugveien 1-3, 0484 Oslo
Telefon: 22845511
E-post: post@helseforskning.etikkom.no
Web: http://helseforskning.etikkom.no/

Komiteen mottok prosjektgruppens tilbakemelding 14.05.2013.

Prosjektet er behandlet på nytt i møtet 13.06.2013.

Skjelettutvalgets uttalelse
Skjelettutvalget har avgitt en grundig vurdering av prosjektet, og identifiserer i uttalelsen tre sentrale forskningsetiske problemstillinger. Det første aspektet har med personidentifisering å gjøre, og utvalget uttaler: *Det er en forutsetning for at prøvene kan benyttes til forskning at de kan knyttes til detaljerte kilder med personidentifiserende informasjon. Hensyn til personers ettermæle, og til eventuelle etterkommere, gjør det til et sentralt anliggende å sikre at slik informasjon ikke kommer på avveie. Forskerne viser til at de vil operere med strenge prosedyrer for å sikre seg mot dette, og dette etiske aspektet ved prosjektet virker godt ivaretatt.*

Det andre aspektet utvalget tar opp, dreier seg om samtykkeproblematikken. Det er på det rene at det ikke foreligger noe samtykke fra pasientene til oppbevaring og fremtidig forskning på deres prøver og legeme. Utvalget uttaler at det anses som lite hensiktsmessig å forsøke å innhente samtykke fra eventuelle slektninger, men kommenterer samtidig at prosjektgruppen trolig overvurderer i hvor stor grad det har vært snakk om et presumptivt samtykke fra pasientene det gjelder: *Søkerne synes å anta at det foreligger en form for antatt samtykke fordi pasientene sannsynligvis "var klar over at materialet skulle brukes til forskning". Det er ikke innlysende at resonnementet er korrekt. At pasientene sannsynligvis forsto at materialet skulle benyttes til forskning, impliserer ikke at de ga sitt implisitte samtykke til dette.* Utvalget konkluderer – på dette punktet – med at det ikke er grunnlag for å si at det foreligger et presumert samtykke.

Den tredje forskningsetiske problemstillingen utvalget peker på angår forbruk av det historiske materialet: *Vi etterlyser en diskusjon av hvor mye av materialet som er tenkt benyttet og hvorvidt det er av verdi å bevare mest mulig for eventuelle fremtidige prosjekter.*

Skjelettutvalget konkluderer på følgende måte: *Som samlet konklusjon finner utvalget, ut fra de dimensjoner ved prosjektet som utvalget har vurdert, at prosjektet kan tilrådes gjennomført forutsatt den nevnte modifikasjonen av den etiske begrunnelsen mht. samtykkeproblematikken, samt at det inkluderes en diskusjon av materialets verdi utover det aktuelle prosjektet.*

Prosjektgruppens tilbakemelding
Prosjektgruppen gir i sin tilbakemelding en ytterligere presisering av tiden og omstendighetene rundt Dr. Gjessings innsamling. I likhet med uttalelsen fra skjelettutvalget anføres det også her at det vil være vanskelig å si noe bastant i forhold til graden av kjennskap pasientene hadde til den forskningen som ble gjort ved Dikemark. I forlengelsen av samtykkeproblematikken understrekes det også at det trolig vil være svært få gjenlevende slektninger av pasientene, og at det ville synes urimelig å be disse om et samtykke så mange år senere.

Tilbakemeldingen klargjør både hjernene og urinprøvenes status i forhold til museumssamlingen ved Dikemark. Materialet er ikke en del av utstillingen, og fra et museumsfaglig ståsted vil det kun være enkelteksempler som er av interesse; det er ikke avgjørende at materialet vises samlet.

Tilbakemeldingen inneholder for øvrig dokumentasjon i forhold til hjemmelsgrunnlaget for obduksjon og oppbevaring av materialet, uten at man har klart å identifisere en egen forskrift for praksisen i perioden 1920-1940.

Komiteens vurdering
Komiteen har mottatt to grundige uttalelser om bruken av materialet.

Når det gjelder spørsmålet om bruken av materialet, og respekt for de personene de gjelder, er dette en vanskelig avveining, uten noe klart fasitsvar. Det er heller ikke mulig å si med sikkerhet hvor mye pasientene visste om den forskningen som foregikk ved Dikemark. At pasientene var behjelpelige med prøveinnsamling kan bety at de kjente til forskningen, men det kan likeledes bety at de var oppsatt på å hjelpe til eller at de anså aktiviteten som behandling. Det er uansett et langt sprang fra velvillig avgivelse av

urinprøver, til oppbevaring av hjerner etter en personens død.

Samtidig er det et faktum at hjernene (og øvrig prøvemateriale) allerede har blitt oppbevart i nærmere 70 år. Komiteen tror ikke det ville krenke personene det gjelder mer at prøvene benyttes til potensiell nyttig forskning, enn at de har ligget i et avstengt museumsrom på Dikemark.

Det er et hovedpoeng ved søknaden at materialet gir en unik mulighet til å studere alvorlig psykisk sykdom og hjerneforandringer fra en tid hvor man ikke medikamentelt behandlet slike tilstander. Komiteen mener det vitenskaplige og samfunnsmessige potensialet ved den forskningen man nå søker om, er betydelig.

Basert på både Skjelettutvalgets innstilling, og prosjektgruppens tilbakemelding, synes det ikke realistisk å henvende seg til eventuelle etterkommere med tanke på innhenting av samtykke. Spørsmålet om bruk av journalopplysningene for pasientene, for tiden lagret i Byarkivet, blir således et spørsmål om dispensasjon fra taushetsplikt. Journalmaterialet er i komiteens øyne å betrakte som opplysninger innsamlet i helsetjenesten.

I henhold til helseforskningslovens § 35 kan REK bestemme at helseopplysninger kan eller skal gis fra helsepersonell til bruk i forskning, og at det kan skje uten hinder av taushetsplikt. Det samme gjelder opplysninger innsamlet i helsetjenesten. Dette kan bare skje dersom slik forskning er av vesentlig interesse for samfunnet, og hensynet til deltakernes velferd og integritet er ivaretatt.

Bestemmelsen må tolkes tilsvarende helseforskningslovens §§ 15 annet ledd og 28 første ledd. I praksis betyr dette at det også skal være vanskelig å innhente nytt samtykke.

Basert på den ovennevnte vurderingen har komiteen besluttet å gi dispensasjon fra taushetsplikt. Helseopplysninger fra de aktuelle personene som skal inkluderes i prosjektet, kan gis uten hinder av taushetsplikt, jf. helseforskningslovens § 35.

Vedrørende videre bruk/oppbevaring av materialet
Det skal opprettes en spesifikk forskningsbiobank for det humant biologisk materialet som vil benyttes i dette prosjektet. Materialet det er snakk om består i tillegg til hjerner, av urin- og avføringsprøver.

Som nevnt ovenfor er det av avgjørende betydning for komiteens behandling av studien, at det dreier seg om et unikt materiale også medisinsk sett. En samling som dette kan romme muligheter for forskning også i fremtiden.

Når det gjelder videre bruk av materialet, legger komiteen til grunn at det er etablert et formalisert samarbeid mellom Dikemark og Oslo Universitetssykehus om overføring av materialet. Spørsmålet om hvor mye av materialet som skal overføres fra Dikemark til forskningsbiobanken tilknyttet prosjektet, anses dermed som et internt anliggende.

Når det gjelder destruksjon av materialet, angis det i hovedsøknaden at materialet blir destruert ved prosjektslutt.

Komiteen aksepterer ikke en slik løsning for dette materialet, jf. helseforskningslovens § 30.

Komiteen har besluttet å sette en lang tidsangivelse for den spesifikke forskningsbiobanken, frem til og med 31.12.2030.

Det er en forutsetning for denne godkjenningen at prosjektgruppen i løpet av denne perioden utarbeider en plan for videre håndtering av materialet, også utover den angitte oppbevaringstiden. Planen skal berede grunnen for en mer varig oppbevaringsløsning – ved for eksempel at forskningsbiobanken søkes omgjort fra spesifikk til generell – og det skal også legges til rette for deling med andre forskningsmiljøer i fremtiden.

Disse vilkårene må ses i lys av den historiske verdien materialet har.

Forskningsbiobankens formalia
Det søkes om å opprette en spesifikk forskningsbiobank med navn *Dr. Gjessings biobank* i prosjektet.

Ansvarshavende for forskningsbiobanken er Ole A. Andreassen. Forskningsansvarlig er Oslo Universitetssykehus.

Forskningsbiobanken vil bestå av hjerner, urin- og avføringsprøver.

Biobanken er gitt en tidsavgrensning frem til og med 31.12.2030. Deretter skal materialet behandles i henhold til de vilkår som er satt for prosjektet, og i henhold til helseforskningslovens § 30.

Det vil ikke være aktuelt med utførsel av materialet til utlandet. Dersom slik utførsel blir aktuelt vil dette kreve søknad til REK, jf. Helseforskningslovens § 29.

Ut fra dette setter komiteen følgende vilkår for prosjektet:
1. Det gis ikke adgang til å destruere det humant biologiske materialet ved prosjektslutt.
2. Det skal utarbeides en plan for videre oppbevaring av materialet etter prosjektslutt.
3. Planen skal legge til rette for deling av materialet med andre miljøer og forskere.

Vedtak
Prosjektet godkjennes med hjemmel i helseforskningslovens §§ 9 og 33, under forutsetning av at ovennevnte vilkår oppfylles.

Godkjenningen omfatter dispensasjon fra taushetsplikt for bruk av helseopplysninger i tråd med søknaden, jf. helseforskningslovens § 35.

Komiteen godkjenner opprettelse av forskningsbiobanken Dr. Gjessings hjerner, i tråd med det som er angitt i prosjektsøknaden. Biobankregisteret vil bli underrettet ved kopi av dette brev.

I tillegg til vilkår som fremgår av dette vedtaket, er tillatelsen gitt under forutsetning av at prosjektet gjennomføres slik det er beskrevet i søknaden og protokollen, og de bestemmelser som følger av helseforskningsloven med forskrifter.

Tillatelsen gjelder til 31.12.2030. Opplysningene skal deretter slettes eller anonymiseres, senest innen et halvt år fra denne dato. For den spesifikke forskningsbiobanken vises det til godkjenningens vilkår.

Komiteens avgjørelse var enstemmig.

Sluttmelding og søknad om prosjektendring
Prosjektleder skal sende sluttmelding til REK sør-øst på eget skjema, jf. hfl. 12. Prosjektleder skal sende søknad om prosjektendring til REK sør-øst dersom det skal gjøres vesentlige endringer i forhold til de opplysninger som er gitt i søknaden, jf. hfl. § 11.

Komiteens vedtak kan påklages til Den nasjonale forskningsetiske komité for medisin og helsefag, jf. helseforskningsloven § 10, 3 ledd og forvaltningsloven § 28. En eventuell klage sendes til REK sør-øst C.

Klagefristen er tre uker fra mottak av dette brevet, jf. forvaltningsloven § 29.

Vi ber om at alle henvendelser sendes inn via vår saksportal: http://helseforskning.etikkom.no eller på e-post til: post@helseforskning.etikkom.no

Vennligst oppgi vårt referansenummer i korrespondansen.